

hairs are simple or forked. Stem of antenna without a branched hair, smooth and without short spines. Apical hair of antenna simple and long. Close to base of antenna starts a thin simple hair which may be taken as the basal hair of antenna.

Shoulder-hairs, internal hair 4-branched, root not prominent, median hair 6 to 9 branched, root conspicuous.

Palmate hair absent on thorax, present on segments 2 to 7 of abdomen. Leaflet of palmate hair lanceolate with a few indistinct serrations, filament is absent and the blade tapers straight to the tip (Plate LVIII, fig 6).

Lateral branched hairs of abdomen present on segments 1 to 6. Very characteristic triradiate spines (Plate LVIII, fig 5) are present on each of the abdominal segments 1 to 7, one pair dorsally and one pair ventrally on each segment as originally pointed out by Christophers (1916).

### 3 *Larva of Anopheles asiaticus.*

All the clypeal hairs are thick, simple and unbranched. The posterior clypeal hairs are equal to or slightly longer than the external clypeal hair (Plate LIX, fig 1). Frontal hairs are very poorly developed and inconspicuous. Both the inner and median frontal hairs are unbranched, while the external hair has 4 to 5 thin branches. Antenna has a branched hair on stem with 4 to 6, sometimes fewer, branches (Plate LIX, fig 2). The apical hair on antenna is simple. Spines on shaft of antenna not prominent. Basal hair of antenna is very characteristically long and simple (Plate LIX, fig 2). Occipital hairs are minute, simple or forked.

Shoulder-hair internal hair 3 to 4 branched, root absent, median hair 12 branches, root conspicuous.

Partially developed palmate hairs on thorax with about 12 leaflets (Plate LIX, fig 5). Palmate hairs present on segments 3 to 7. Palmate hair leaflet is spindle shaped, with a few sharp serrations and a long filament ending acuminate at tip (Plate LIX, figs 3 and 4). Branched lateral hairs of abdomen present on segments 1 to 7. Body setæ absent.

### 4 *Larva of Anopheles annandalei.*

The following is a description of the mature larva of *Anopheles annandalei*, which is based on a study of over forty mature specimens collected at Sukna, Darjeeling district. The previous description (Iyengar, 1922) was based on three immature larvæ that were the only specimens known at the time.

Internal clypeal hairs are long and simple and usually do not show any fraying. The external clypeal hairs have 4 to 7 branches (Plate LIX, fig 7). The posterior clypeal hairs are very short and simple.

Frontal hairs are small and poorly developed. Inner frontal hairs are simple and long, the tip extending as far as the bases of the clypeal hairs. The other frontal hairs are short and have 2 to 4 branches. Occipital hairs are simple and minute. The inner occipital hairs is frequently forked at apex. Antenna without a branched hair on stem. Spines on stem of antenna short and not well

developed Basal hair of antenna long and with 6 to 8 pinnate branches Apical hair of antenna branched

Shoulder-hairs internal hair with 10 to 13 branches, root not well developed, median hair with a very prominent root and 14 to 19 branches External shoulder-hair starts close to base of median hair and is simple

Palmate hairs present on thorax\* cockade-like, not well developed and with about 8 to 10, occasionally more, leaflets (Plate LIX, fig 9) Palmate hairs absent on 1st abdominal segment Palmate hairs present on segments 2 to 7 of abdomen Number of leaflets usually between 16 and 20 on each palmate hair Palmate hair leaflet moderately broad, and gradually tapering, serrations minute, filament absent (Plate LIX, fig 6) The leaflet looks very much like the leaflet of *A barbuostis* or *A lycanus*

Large lateral branched hairs occur on abdominal segments 1 to 6 (Plate LVIII, fig 1) Short dark curved setae present on ventral and lateral aspect of the integument of thorax and abdomen (Plate LIX, fig 8) The setae are short measuring from 12 to 16  $\mu$  long They project forwards in the thorax and backwards in the abdomen

#### 5 Larva of *A annandalei* var *djajasanensis*

Clypeal hairs internal hairs thick and long, simple, external hair, thick, 6 to 8 (sometimes more) branches, the branches being close to apex posterior clypeal hair short and simple, about half-way between the internal and external hairs

Frontal hairs are poorly developed, internal hairs simple and long, occasionally bi-fid, median ones short and thin, simple or 2- to 3-fid, external hairs thin and 4 to 7 branched All the occipital hairs are simple

Antenna without a branched hair on stem Shaft of antenna with only minute spines Basal hair of antenna long and with 5 to 7 pinnate branches

Shoulder-hairs internal hair with 12 to 15 branches, root not developed, median hair with a large root and 14 to 21 branches, outer hair starts from a cleft in the root of the median hair † Tip of outer shoulder hair is simple (or minutely 2- or 3-fid in very young specimens)

Palmate hairs a pair of conspicuous cockade-like palmate hairs occur on thorax, palmate hairs occur on abdominal segments 2 to 7 Leaflet of palmate hair as in Plate LIX, fig 10, the serrations are abrupt and conspicuous, the blade is not broad, the tip of the blade is elongate and blunt, filament absent

Lateral branched hairs of abdomen present on segments 1 to 7 Ventral and lateral aspect of thorax and abdomen thickly bristled over with numerous

\* Iyengar (1922) in his description of *A annandalei* larva had missed transparent palmate hairs on the thorax

† Brug (1926) has observed that the external shoulder-hair starts from a small root which lies hidden in a sinus in the root of the middle hair, so that it sometimes appears as if it arises from the root of the middle hair 'Het-buitenste haar ontspringt van een kleinen wortel, die een sinus van den wortel van het midden haar verscholen ligt, zoodat het soms schijnt alsof het uit den wortel van het laatste haar ontspring'



closely placed long and curved setæ which are very conspicuous and give the skin a brush-like appearance (Plate LIX, fig 11) The setæ measure about 28 to 32  $\mu$  long

The following synoptic table has been drawn up to differentiate between the larvæ of the oriental tree-hole breeding species

- |   |   |  |
|---|---|--|
| 1 | Triradiate spines present on abdominal segments 1 to 7, one pair dorsally and one pair ventrally, basal hair of antenna not conspicuous, palmate hair absent on thorax  | <i>barianensis</i>                           |
|   | Triradiate spines of abdomen absent, palmate hair usually present on thorax   | 2  |
| 2 | Clypeal hairs very thin, posterior clypeals external to outer clypeals, sub-antennal hair characteristically clubbed  | <i>culiciformis</i>                          |
|   | Clypeal hairs not thin, posterior clypeals internal to outer clypeals, basal hair of antenna not clubbed  | 3  |
| 3 | External clypeal hair simple, posterior clypeal quite long, basal hair of antenna simple, antenna with branched hair on stem, palmate hair leaflet with a long and thin filament, ventral and lateral aspect of abdomen and thorax not covered with minute setæ | <i>asiaticus</i>                             |
|   | External clypeal hair branched posterior clypeal very short, basal hair of antenna branched, antenna without a branched hair on stem, palmate hair leaflet without a long filament, ventral and lateral aspect of thorax and abdomen shagreened                 | 4  |
| 4 | Serrations of palmate hair leaflet feeble and minute, blade very gradually tapering to tip, body setæ short, thin and sparse  | <i>annandalei</i> *                          |
|   | Serrations of palmate hair leaflet conspicuous and abrupt, blade abruptly tapering by deep serrations, body setæ long, thick and densely distributed on integument  | <i>annandalei</i> var <i>djajasanensis</i> * |

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\*Puri (1929) in a paper which has been published while the present one was in press, has differentiated the specimens of *A annandalei* collected from tree-holes in the Eastern Himalayas and Khasi Hills into a type form and a new variety, i.e., *A annandalei* var *interruptus* Puri. He has placed the Java form, i.e., var *djajasanensis* Brug as a synonym of the type form, *A annandalei* Prashad. I do not agree with the views expressed by Puri in that paper (*Ind Jour Med Res*, Vol XVII, No 2, Oct, pp 385—396)

The chief characters in general of the larvæ of the tree-hole breeding Anophelines were enumerated at an earlier part of the paper and may be briefly summarized as follows. Clypeal hairs usually unbranched, frontal hairs poorly developed and inconspicuous, occipital hairs thin and minute, short spines on shaft of antenna absent or poorly developed, basal hair of antenna usually present, lateral branched hairs of abdomen present on segments 1 to 6.

Two other tree-hole breeding species of *Anopheles*, known from other parts of the world namely *A. plumbeus* Hal, from Europe and *A. barberi* Coq, from North America, also exhibit the above-mentioned general characteristics of the oriental tree-hole breeding Anophelines.

*Anopheles barberi* and *A. plumbeus* together with the oriental *A. culiciformis* and *A. bharuensis* were originally placed under Genus *Coelodiazesis* D & K. These four species possess several common features both in adult and larval stages and they form a small natural group of allied species. *Anopheles asiaticus* and *A. annandali* belong to a different group on account of their spotted wings and the presence of the dense scale tuft on the femoro-tibial joint in the adult. These two species were placed under Genus *Lophoschelosmyia* Theob. Although both of these genera were subsequently sunk to subgeneric rank and finally sunk under Genus *Anopheles* subgenus *Anopheles* (Christophers, 1924), these two groups are quite distinct in their adult stage and could even be differentiated in the larval stage. In the *Coelodiazesis* group, the larva has extremely thin, inconspicuous and always unbranched clypeal hairs, and the antenna is almost entirely devoid of spines on stem. In the *Lophoschelosmyia* group, the clypeal hairs are thick, the outer ones are occasionally branched and the antenna has minute spines on the shaft.

In both of these groups, in spite of wide differences in the adult stage, the larvæ are very much alike and the characteristic features of the tree-hole breeders noted above occur without an exception. These characters appear to be due more to a convergence, resultant on a similar habitat, rather than to any close affinity, since it is seen that there is very little affinity between the two groups, their adults being very different. Characters such as the reduction of frontal hairs and extra development of branched lateral hairs of abdomen are possibly the effect of the peculiar type of habitat. But it must be confessed that the ecological usefulness of such characters to the species is, however, a matter for conjecture.

Edwards (1922) says with reference to *Anopheles plumbeus*, 'The tree-holes in which these larvæ are found are frequently (perhaps usually) dark and it may be supposed that the sense of sight would be of little value to the inhabitants of such places, while that of touch would attain a greatly enhanced importance, since the mosquito larvæ have a number of predaceous enemies living with them. Possibly this may help to explain the development of hairs on the soft parts of the body though their reduction on the head is not so easy to account for. The development of additional chitinous plates may be merely a chemico-physical reaction to the excess of tannin in the water.' Edwards thinks that 'whatever may

be the use of these larval modifications, it seems certain that they are due to environmental conditions' Since these characters occur in very different groups of Anophelines occurring in very widely separated areas, there can be little doubt that these larval characters illustrate a convergence of character resulting from the peculiar habitat

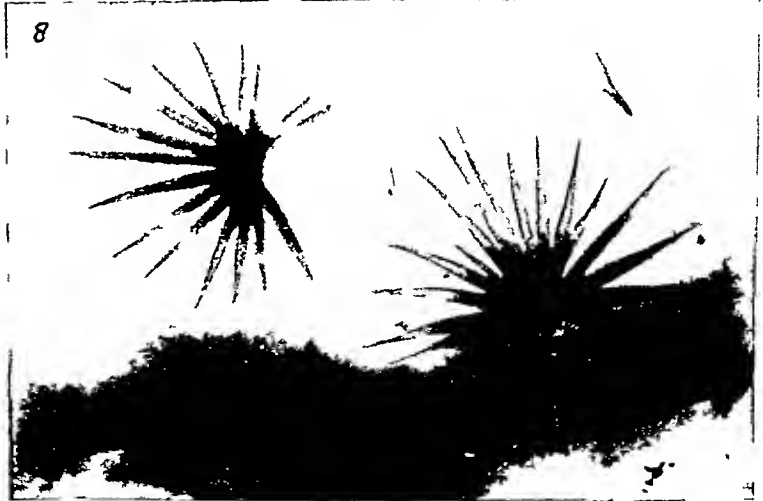
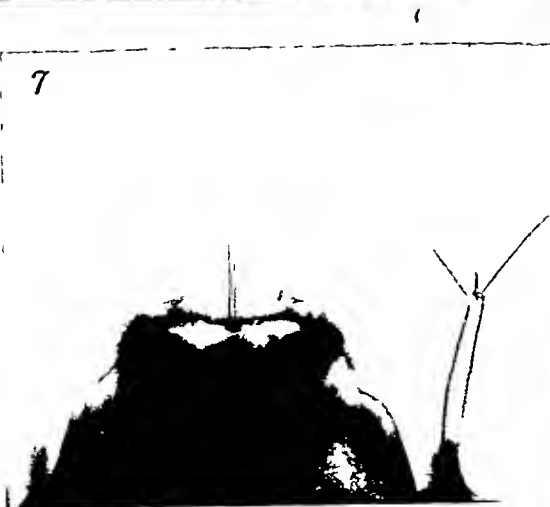
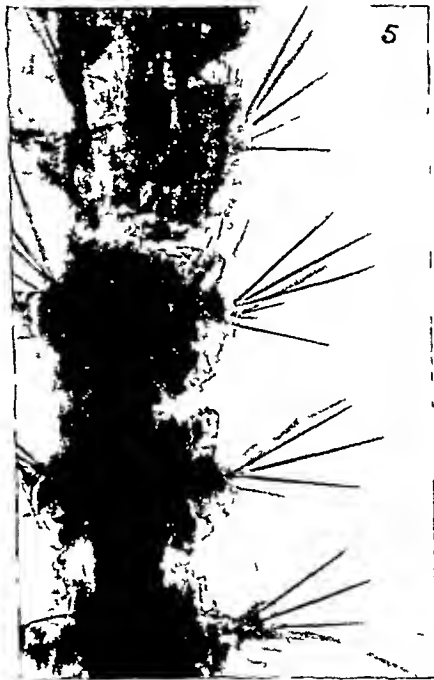
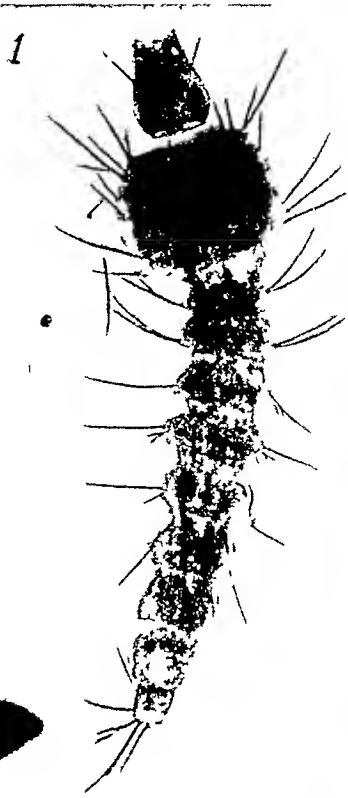
## ACKNOWLEDGMENTS

The material for study was obtained through the courtesy of several workers to whom I am greatly indebted. Lieut-Col S R Christophers, F R S, gave me specimens of *A. bariensis* and *culiciformis* in 1922, and Mr P J Barraud gave me a larva of *A. annandalei* at about the same time. To Dr B A R Gater, I am indebted for specimens of *A. asiaticus*, and to Col S L Brug for specimens of *A. annandalei* var *djajasanensis*. I take this opportunity of thanking them for their kind help.

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### EXPLANATION OF PLATE LVIII

- Fig 1 Larva of *Anopheles annandalei* showing the branched lateral hairs on abdominal segments 1 to 6
- „ 2 Head of *Anopheles annandalei* showing the poorly-developed frontal hairs
- „ 3 The frontal hairs of the normal type are well developed, branched and feather-like Head of larva of *A. varuna* showing the well-developed frontal and occipital hairs
- „ 4 Clypeal hairs of *A. bianensis* The outer clypeal hair of the left side is forked which is not a normal condition
- „ 5 Lateral triradiate spines on the abdomen of *A. bianensis* larva Each of the abdominal segments 1 to 7 have a pair of such triradiate spines dorsally and another pair ventrally
- „ 6 Palmate hair of *A. bianensis* (5th segment)
- „ 7 Head of *A. culiciformis* showing clypeal hairs and antenna The antenna is nearly devoid of spines and the apical hair is long and simple
- „ 8 Palmate hairs (4th and 5th segments) of *A. culiciformis* larva

### EXPLANATION OF PLATE LIX

Figs 1 to 5, *A asiaticus* larva, figs 6 to 9, *A annandalei*, and figs 10 to 11,  
var *djajasanensis*

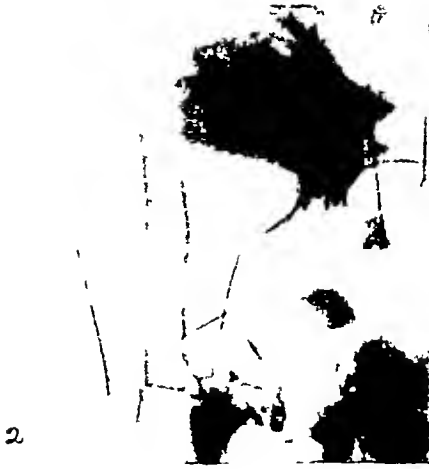
- Fig 1 Head of *A asiaticus* larva showing clypeal hairs The unbranched frontal hairs of the right side are also visible
- „ 2 *A asiaticus* larva antenna showing branched hair on stem, basal hair of antenna is long and simple
- „ 3 Palmate hairs of *A asiaticus* larva (segments 3 to 7)
- „ 4 Palmate hairs of *A asiaticus* larva (segments 6 to 4)
- „ 5 Thoracic palmate hairs *A asiaticus*
- „ 6 *A annandalei* larva, palmate hair of 5th segment
- „ 7 *A annandalei*, clypeal hairs
- „ 8 *A annandalei*, part of thorax showing body setæ
- „ 9 Thoracic palmate hair of *A annandalei*
- „ 10 Palmate hair (5th segment) of *A annandalei* var *djajasanensis*
- „ 11 Dense shagreening of body of var *djajasanensis*.

PLATE LIX

1



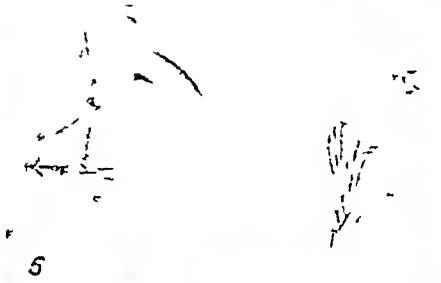
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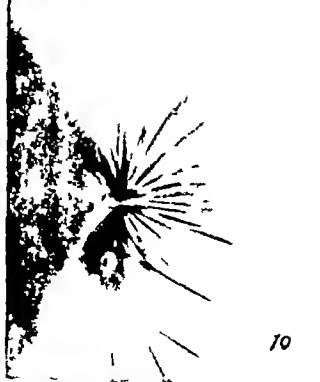
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# STUDIES IN 'PERNICIOUS ANÆMIA' OF PREGNANCY

## Part I.

### PRELIMINARY REPORT

BY

LUCY WILLS, M A (Cantab), M D, B S (Lond), M R C S, L R C P,

AND

MANECK M MEHTA, M A, Ph D, D S C (Lond),  
*Maternal Mortality Inquiry Indian Research Fund Association,  
Haffkine Institute, Bombay*

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THE condition described as pernicious anæmia of pregnancy is a severe anæmia of unknown origin with a blood picture of the pernicious type, which occurs in pregnant women, sometimes in successive pregnancies, and is frequently fatal at term. It is well known in India and has been described by Balfour(1) and McSwiney(2). It occurs in all communities, Hindu, Mahommedan, Parsee, Christian (Goanese and other Indian Christians) and Beni Israel, but is comparatively rare in Europeans. A similar condition has been described in Europe(3) and America(4 and 5), but in these countries it is a rare complication of pregnancy and from the published accounts it would seem to be a different entity to the condition prevailing in India. Dr Summerhayes, in a personal communication, describes what would appear to be an identical condition from Accra on the Gold Coast where cases occur among the patients admitted to the Native Maternity Hospital. As there is no full account of these latter cases and as the European and American ones differ from the Indian ones, this paper will be limited to a description of the disease as it occurs in India.

Balfour's and McSwiney's papers should be referred to for details of the clinical picture, but certain points must be briefly reviewed here, as before any description of the blood and other pathological findings can be undertaken, it is essential to have a clear conception of the entity with which we are dealing. The history and clinical features of the condition are also of importance in relation

to its differential diagnosis from true pernicious or Addison's anæmia and to a consideration of the possible ætiology of the disease

#### SELECTION OF CASES

All anæmic cases admitted to the maternity wards of the Cama and Albles Hospital and to the Wadia Hospital were referred to us. From these we excluded cases secondary to hæmorrhage, tuberculosis and similar causes, leaving a group whose blood picture more or less closely resembled that of true pernicious anæmia. To simplify the issue further all cases were excluded that were found to be suffering from malaria, hookworm disease, syphilis or severe nephritis—conditions known to produce, in certain instances, a blood picture resembling that of pernicious anæmia. In a series of 66 consecutive cases seen in six

years (October to March, 1928-29), 16, or 24 per cent, were thus complicated, so excluded, leaving a group of 50 cases of so-called 'idiopathic' pernicious anæmia of pregnancy. These 16 cases were excluded on the finding of malarial or hookworm parasites, a positive Kahn's test, or signs or symptoms of nephritis respectively, but most could have been separated on a detailed study of the blood picture, it is hoped to describe them later as they are of considerable interest.

It is possible that malaria was missed in certain cases, but the bloods were searched fully and, when obtained, the placentas also. The majority of the remaining 50 cases were first seen in the latter months of pregnancy, a few were examined as early as the fourth month and the remainder not until immediately after delivery. It is extremely difficult to determine the time of onset of the disease as the patients give very vague histories, but many of the post-partum cases were undoubtedly anæmic before delivery though no count had been made, whereas others seemed to develop a severe anæmia immediately after delivery, the latter were probably secondary to sepsis.

Besides 50 pregnant cases 5 non-pregnant women were admitted who, save for the accident of being non-pregnant, presented an exactly similar clinical and pathological picture. These 5 cases will, therefore, be considered with the 50 pregnant cases. It is probable that non-pregnant cases are far more frequent than these figures would suggest, as it must be remembered that this work was done in lying-in wards and non-pregnant cases were rarely admitted.

#### GENERAL DESCRIPTION OF IDIOPATHIC CASES

The anæmic cases, selected in the way described above, are characterized by the severity of the anæmia and the paucity of signs and symptoms other than those secondary to any severe anæmia. Oedema of the feet and ankles, puffiness of the face, weakness, low blood pressure and a history of fever at some time during the disease are the most constant findings. It is very remarkable how active many of these patients are in spite of extreme degrees of anæmia, some patients actually walking up to hospital with red cell counts of half-a-million. In the above respects the condition resembles Addison's anæmia as also in the

frequency of intestinal disfunction (diarrhoea, vomiting, sore mouth and tongue) The frequency and severity of the diarrhoea is very variable and the fact, personally reported by Dr Margaret Balfour, that it is far more frequent and fatal in certain seasons, would suggest an intercurrent secondary infection

Certain characteristics of this pregnancy anaemia differentiate it from true pernicious anaemia of these the age and sex incidence, the course, either a fatal issue at or near term, or frequently a rapid recovery after delivery, and the absence of the characteristic lemon-yellow colour and jaundiced sclerotics, are the most striking

The disease appears to be seasonal in Bombay, most of the cases occurring between October and March. The same incidence is reported by McSwiney from Calcutta (personal communication). In Bombay there are several factors, such as the seasonal variation in the birth-rate, the annual exodus to the country and possibly the climatic conditions, which must be considered in relation to this time distribution, but further work along these lines is urgently needed. A similar seasonal incidences of true pernicious anaemia has been reported by Bartlett(6) who noted in Pennsylvania that it had a quite definite incidence in the spring and summer

Unfortunately no post-mortem could be obtained in the present series, so the morbid anatomy of the condition has not been studied and cannot be compared with that of pernicious anaemia

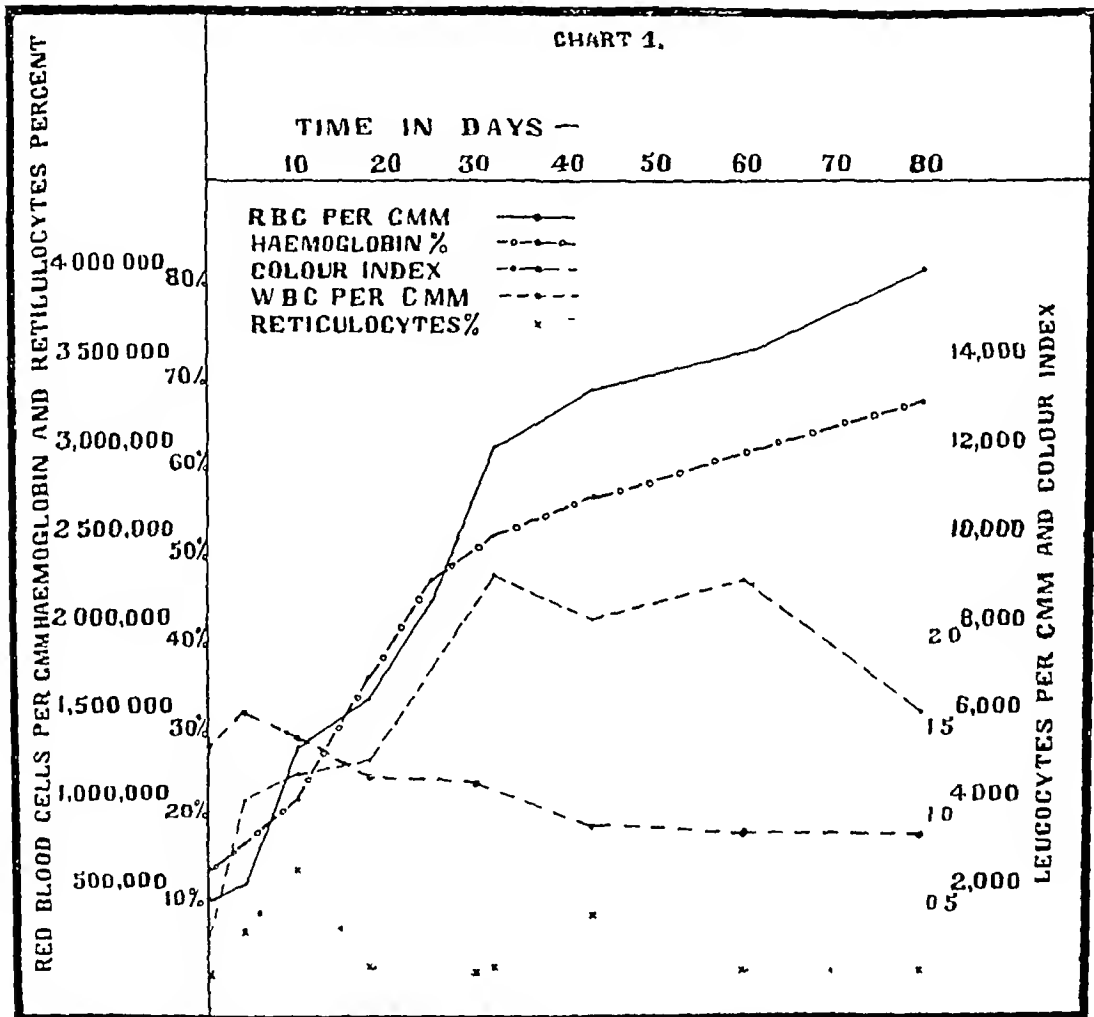
### THE BLOOD

It is important in all blood examinations to consider the stage of the disease under consideration, this is particularly so in the present instance, as very marked and rapidly arising differences occur in the counts and chemical findings as the disease progresses either to a fatal termination or to a cure. Protocols I and II record the blood findings at first examination, that is in untreated cases, and when active regeneration is taking place. Charts 1 and 2 show the characteristic changes graphically

(a) *Red cells, hæmoglobin and colour index* — These will be considered first in active, untreated cases, which may be either ante- or post-partum, or non-pregnant. The anaemia is typically a macrocytic one, the degree of anisocytosis varying broadly with the degree of anaemia. Poikilocytosis is not marked, and in this respect these anaemias differ from true pernicious anaemia. There is little or no polychromatophilia and the number of reticulocytes is low. Nuclear forms do occur but are infrequent, except in a few cases when there is a shower of normoblasts, true megaloblasts are found but normoblasts predominate

Protocol II shows the change that occurs when recovery, either spontaneous or more frequently as a result of treatment with liver extract, is beginning and active blood regeneration is taking place. There is a marked anisocytosis with the presence of many polychromatic cells and cells showing punctate basophilia, polychromatophilia being frequently very marked in the macrocytes

Nucleated forms occur sometimes in large numbers but again normoblasts predominate. The reticulocytes increase enormously, but there is a lag in the reticulocyte response, the maximum being obtained generally several days after the other signs of regeneration mentioned above have passed their maximum development. The response to liver treatment described above is exactly similar to that seen in treated cases of true pernicious anæmia.

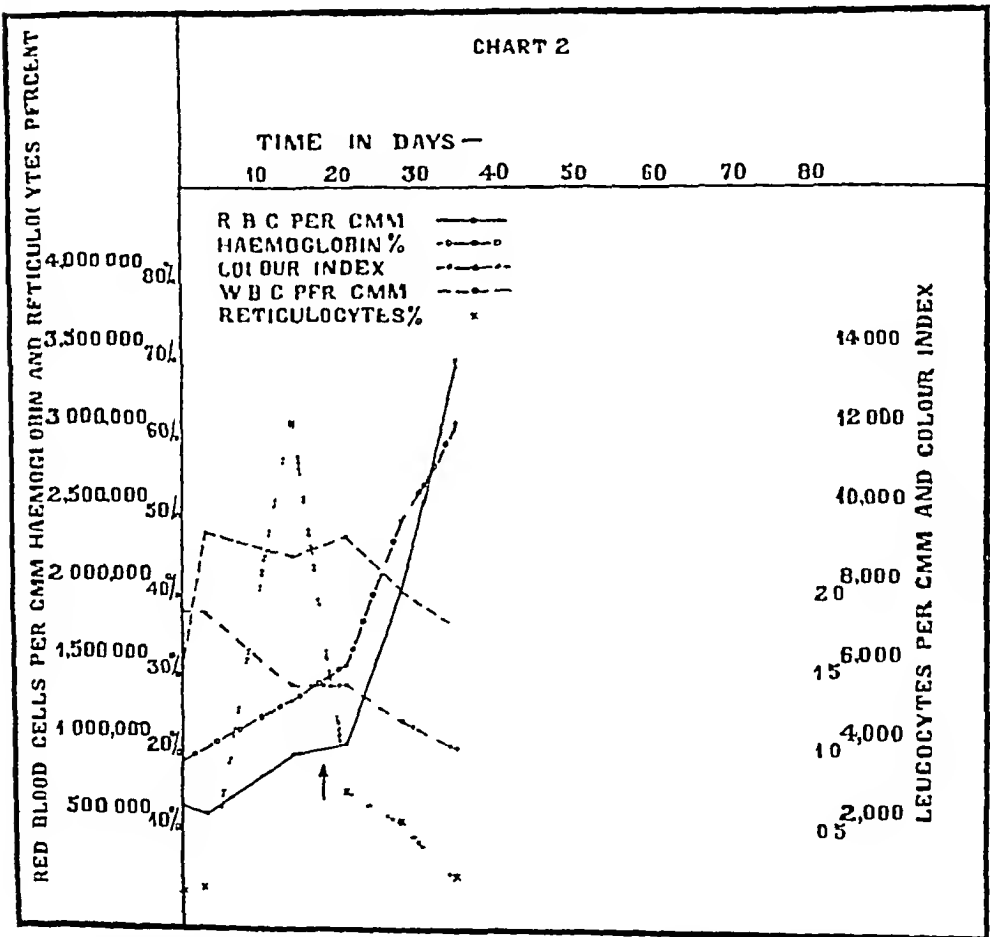


**CASE 6 NON-PREGNANT, TREATED LIVER EXTRACT FROM FIRST DAY**

The presence of large hæmoglobiniferous cells gives a high colour index of 1.0 or more in the untreated cases and at the beginning of treatment, but as regeneration takes place the output of red cells runs ahead of the increase in hæmoglobin and the number of large cells also decreases, so that the colour index very rapidly falls below unity and values of 0.8 and 0.75 may be obtained.

The response to liver treatment, as stated above, is good, but in the pregnant or post-partum cases the red cell counts and hæmoglobin values seldom reach

figures that could be considered normal in a European woman. This was particularly noticeable in the undelivered cases, where the highest count obtained was 3,250,000 per cmm. These findings are partly due to the time factor as so few cases present themselves for treatment early in pregnancy, hence there is not enough time before delivery for high figures to be reached, but the extra demands made during pregnancy on the blood-forming organs must also, in our opinion be taken into consideration. In my case a count of 3,000,000 was considered satisfactory, as many so-called healthy women of the same class have similar counts.



CASE 25 PREGNANT. DELIVERED AT ↑ LIVER EXTRACT FROM FIRST DAY.

The low colour index found in treated cases has to be borne in mind when considering mild cases of this condition. Cases were occasionally admitted that could not definitely be diagnosed as 'pernicious anaemia of pregnancy' as the counts were not quite typical and the colour indices were low. Some of these

cases went downhill rapidly, sometimes in the course of a week or ten days, and then presented the typical pernicious-like blood picture, with macrocytosis and a high colour index

(b) *White cell count*—In many of the severe untreated cases the white cell count, as in true pernicious anæmia, is low and the inversion of the polymorphs and lymphocytes, typical of the latter disease, is also present. The reduction in the number of white cells is the more remarkable as in normal pregnancy there is a slight leucocytosis. Many of the cases, however, do not show this characteristic leucopænia but have either normal or raised counts, especially is this so in the post-partum cases where the raised values are nearly always associated with some degree of sepsis and show a polymorphonuclear leucocytosis. Besides the relative increase in lymphocytes, myelocytes or even myeloblasts may be present, the former sometimes in very appreciable numbers.

When blood regeneration starts, the white picture changes very noticeably. Typically there is an increase in the total count but the characteristic feature, when the initial response to treatment is good, is the change in the granular cells. Numerous myelocytes and metamyelocytes appear and there is a marked increase in the total and relative number of granulocytes, so that the characteristic inversion of the untreated cases rapidly disappears. As improvement continues the white cells decrease again in number and the immature granular cells disappear from the blood stream.

(c) *Platelets*—The number of platelets is decreased during the active stages of the disease.

(d) *Van den Bergh reaction*—The number of cases in which this reaction was done in the present series is small, so for purposes of comparison the values from another series, previously obtained by Dr Mehta, are included in Protocol III. In the present series the test was repeated at regular intervals during the patient's stay in hospital. Protocol III gives the figures obtained and these show, in contrast to the findings in true pernicious anæmia, that the indirect Van den Bergh reaction is not increased but falls within normal limits. Case 50 had a high value at one determination, but this was made when the patient was jaundiced after severe hæmorrhages from various mucous surfaces. If the cases with a positive Kahn's test or in which malaria was diagnosed had been included, much higher values would have been obtained, a fact which should be borne in mind when the so-called 'idiopathic' cases are under consideration. A high indirect Van den Bergh reaction should suggest undiagnosed malaria or syphilis.

It may be noted here that in 39 cases whose urine was examined urobilin was found only once, whereas in urine from active cases of pernicious anæmia it is constantly present.

(e) *Other blood chemistry*—The number of analysis made was small. Certain findings, however, appear to us to be suggestive though a larger series of figures is necessary before very definite deductions can be made from them. Protocol IV gives the results up to date and includes a small control series obtained

PROTOCOL III  
I an den Beigh

PRESENT SERIES			OLD SERIES		
Case No	Direct units	Indirect units	Case No	Direct units	Indirect units
11		0.1	1		0.5
22			2	1.1	1.1
39			3		0.7
40		2.0	4		0.5
43			5		
44		1.25	6		
46		Very faint	7	2.6	2.0
49		Very faint	8	0.6	0.6
50			9		
	2.0	5.0	10		0.2
	Immediate				
		2.5	12		0.3
			13	0.4	0.6
51		Very faint	1	0.8	0.5
52			2		0.6
54		Very faint	4		
55		0.2	5		
			7		Very faint
56			9		
59			10		0.3
60		1.2	11		0.5
		Very faint	12		
61			13		
62			14		Very faint
64			15		
65	Immediate and delayed	2.0	16		
68	Very faint	Very faint			
69					

Many cases several estimations at weekly intervals, but figures only entered if different on different dates



## PROTOCOL IV

*Chemical analysis of blood.*

Class	Number of analyses	Red cell per c mm blood	Mg per cent, whole blood, average figures			Number of analyses	CALCIUM Mg per cent, serum.	INORGANIC PHOSPHATES Mg per cent, whole blood	
			Non-PROTEIN NITROGEN	Urea	RATIO UREA N NON-PROTEIN NITROGEN				NaCl
Controls .	16	Average 3,566,000	22.7	17.5	39.8	477	11	3.38	
Anæmic all cases .	30	Under 3,000,000	23.1	20.8	45.4	539	51	2.86	
	6	Under 1,000,000	40.9	41.5	47.8	544	9	2.80	
	24	Under 3,000,000	23.3	19.8	44.8	538	42	2.9	
Osteomalacia untreated .	.	..					17	1.54	

from healthy pregnant women of the same class, and a series of untreated osteomalacia cases. If the figures from the series with blood counts under 1 million are excluded, there is no significant difference between the non-protein nitrogen values obtained in the control and in the anæmic cases. In the extreme anæmias there is a slight increase in the total non-protein nitrogen which is largely accounted for by the increase in urea so that the ratio is increased above normal unlike the ratio from the toxæmias of pregnancy which is decreased. The chloride figures from the anæmic series are increased proportionally to the degree of anæmia i.e., to the increase in plasma volume.

It is the calcium and inorganic phosphate figures that are of interest however. In individual cases in the anæmic series, values were found that suggested a condition of latent osteomalacia which was confirmed in several cases by the patients subsequently developing the symptoms of that disease. An examination of the total average figures for both calcium and phosphorus shows that both the anæmic and the osteomalacia series have lower calcium values than the normal and that the anæmic series does not differ significantly from the osteomalacia series whereas the anæmic cases only probably have lower phosphorus than the normal though the osteomalacia value is certainly lower than both the normal and the anæmic values. These findings suggest that in the anæmic series cases occur that are definitely low in both calcium and inorganic phosphorus, presumably due to a lack of vitamin D as in the osteomalacia cases.

#### TEST MEALS

A test meal of 50 cc of 7 per cent alcohol was used throughout. The result of the test meals are recorded in Protocol V. It is at once obvious from the figures that achlorhydria, far from being constantly present as in true pernicious anæmia, is very rarely present in the condition under consideration. In one non-pregnant and three pregnant cases no free acid was found, but as the mineral chlorides were above 40 per cent in all cases, the achlorhydria is of a different order to that found in true pernicious anæmia where the chloride values do not exceed 30 to 35 per cent. The range of acidity and chloride values is similar to that found in any series of hospital patients.

#### CHEMICAL EXAMINATION OF URINE

No abnormality was discovered in the urine with the exception of the presence of a cloud or trace of albumen in 35 per cent of all cases.

#### BACTERIOLOGICAL STUDIES

Emmanuelov and Mehta (7) in previous work from this laboratory, failed to establish the ætiological significance of the presence of either *B. welchii* or *streptococci* in the stools and urine of these cases. In animal experiments with both organisms the typical blood changes were not produced, and the presence of such organisms in the stools even in large numbers has been shown by Davidson (8) in his work on the intestinal flora of pernicious anæmia to have no direct

## PROTOCOL V

*Test meals*

Case No	MAXIMUM FREE ACID ITY	MAXIMUM TOTAL ACID ITY	MAXIMUM NEUTRAL CHLORIDE	Case No	MAXIMUM FREE ACID ITY	MAXIMUM TOTAL ACID- ITY	MAXIMUM NEUTRAL CHLORIDE
	As c c per cent N/10 solution				As c c per cent N/10 solution		
5	40	60	93	56	185	41 5	75
7	40	57	82	62	34	41	93
8	52	62	98	64		7	56
10	22	30	50	A	49	63	105*
11		14	30	B		7	40*
11	28	36	93	D	53	62	102*
20		5	65	E	22	30	73
20	15 5	17 5	76	F	7 5	27	65
26	7	20	25	Non-pregnant			
39	69	85	90	6		10	83
43	14	17	101	6	42 5	55	93
49	17	43 5	58	14		10	92
50	19	29	93	14	17	28	76*
52	16	26	86	30	12	15	60
55		16	65	61		5	55
55		6	68			4 5	93

\* Specimens had bile in them

Test meal used 50 c c 7 per cent alcohol Samples withdrawn every 10 minutes

relationship to the disease. In the present studies, the stools from a small series of cases were fully investigated in Colonel Acton's laboratory at Calcutta. The stools were plated with special reference to the presence of streptococci but though numerous organisms, many pathogenic, were isolated, in only one case was a streptococcus isolated.

Many workers, notably Acton (personal communication), think that this anæmia is caused by a streptococcal or other infection, which originates in the gut but which becomes general owing to changes in the gut-wall allowing the organism to pass into the general circulation. In the cases studied in Bombay, we have failed to find any constant gut infection from an examination of the stools, but as such negative evidence is inconclusive, the greatest attention was paid to the culturing of the urine to see if any evidence of such infection could

be obtained by this means. Balfour(1) had previously failed to find evidence of a blood infection by blood cultures.

In the examination of the urine Aeton's technique was employed throughout. Twenty-eight cases were examined before delivery: the urine was sterile in 25, grew *B. coli* in 2 and a non-hæmolytic streptococcus in 1. The cases examined post-partum yielded positive cultures more frequently as would be expected. Out of 11 cases examined hæmolytic streptococci were found in 2, non-hæmolytic streptococci in 4, a diphtheroid organism in 1 and *B. coli* in one. All but one of the infected cases were suffering from severe diarrhoea. In no case was an anaerobe isolated from the urine (Protocol VI).

#### PROTOCOL VI Results of culture of urine

	PRESENT SERIES		PREVIOUS SERIES *	
	NUMBER OF CASES		NUMBER OF CASES	
	Ante partum	Post partum	Ante partum	Post partum
Sterile	25	3	7	2
Hæmolytic streptococci	0	2	1	3
Non hæmolytic streptococci	1	4	2	2
Other organisms	2	2	0	0

\* Cases from Calcutta and Madras reported by Balfour(1)

Balfour(1) has reported a much higher incidence of urinary infections but if her figures, for a series of cases studied in Madras and Calcutta, are analysed it is clear that the majority of the cases were in women who were examined post-partum. The fact that a large number of the cases occurred in one lying-in hospital, suggests the possibility of an epidemic of puerperal sepsis.

The resting gastric juice was also cultured but neither streptococci nor gut organisms were ever recovered except in one case from which a streptococcus was grown similar to one obtained from the very septic gums. As nearly all the cases had free acid in their resting juice, these results are not unexpected.

Through the kindness of Dr Muriel Robertson of the Lister Institute, London, the serum of several of these patients has been examined against antigens of *B. tetani*, *B. welchii* (3 strains) and *Vibrio septique* but only negative results have been obtained.

#### DIETETIC STUDIES

It was recognized early in the investigation that any inquiry into the diets or home conditions of the patients that depended on statements made by the

patients themselves would be valueless, as the women never give consistent answers to any question. To remove this difficulty the patients were visited after discharge and detailed notes of the actual food eaten, number of persons sharing meal, conditions of house, etc., were made on six consecutive days. From these the average daily diet was calculated. The results are of considerable interest, though their full significance cannot be determined till a much larger series has been collected as well as a control series. But the diets collected have shown strikingly constant errors, the most important of which is a marked deficiency in vitamins A and C, due to the small quantity and poor quality of the fats and vegetables eaten. Many diets were of low caloric value, partly due to the extreme fat-deficiency, which is nearly always associated with an absolute preponderance of carbohydrate, and partly to the generally small food intake. The diet of the better class Mahommedans must be excluded from this generalization, as it is both high in calories and in fat. The fat used is however obviously poor in vitamins as in such households, besides cases of anæmia, well-marked cases of rickets and osteomalacia are encountered. Colorimetric tests for vitamins A and D (to be reported in full later) have also shown that the ghee in such household is of very poor quality. As regards vitamin B there is little evidence of a deficiency except in a few cases, chiefly Goanese whose diet consists largely of polished rice, white bread and fish. As mentioned above, there is frequently an associated D deficiency shown by the presence of osteomalacia and rickets in the same households. Further, several of the patients developed osteomalacia while in hospital and the blood analyses also suggested that the intake of D was low. But the fact that the associated B and D deficiencies only occurred in a limited number of the cases, suggest that these have no direct relationship whereas the constant A and C deficiencies appear to be of ætiological significance.

#### ANIMAL EXPERIMENTS

The material for this work was generously placed at my disposal at Coonoor, by Colonel McCarrison, who had previously, in his report to the Conference of Medical Research Workers at Calcutta in 1927, stated that he had produced in rats on certain experimental diets a condition similar in post-mortem appearances to pernicious anæmia. An examination of the records(9) of these experiments showed that on a diet of oatmeal, cornflour and linseed meal with a mixture of sodium chloride and calcium phosphate, 25 per cent of the rats developed a profound and fatal anæmia. In addition 8 other animals developed a severe anæmia but recovered. In another similar series of experiments, 11 per cent of the animals died from severe anæmia. Many of the animals showed the curious lemon-yellow tint characteristic of pernicious anæmia, but in others the skin was pearly white. The animals were variously housed, some in large and some in small colonies while some lived on screens and some on wooden floors. Anæmia occurred under all these conditions but was slightly more frequent in the large colonies which suggests the possibility of a microbic factor. The addition, however, of 10 c.c. of milk to each individual's ration completely protected control

animals from developing anaemia which would not only suggest that the microbial factor was secondary but that the vitamins A and C contained in the relatively large amount of milk consumed by the animals afforded protection against the anaemia

In the original experiment the greater number of deaths from anaemia occurred in pregnant animals either after the birth of a still-born litter or undelivered. Intra-uterine haemorrhages were noted post-mortem.

An examination of the few films available showed a blood picture resembling that found in human cases where a certain degree of blood regeneration is taking place. The slides showed marked anisocytosis of a macrocytic type, increase in the number of nucleated forms, mainly normoblasts, marked polychromatophilia and punctate basophilia, and a large increase in the granular leucocytes.

The diets producing these anaemias in rats were all deficient to a marked degree in vitamins A and C, but in comparison to the majority of the human diets they have a relatively high fat content. When fresh milk is added to these diets, and animal protein added also, the A and C deficiencies are corrected.

### DISCUSSION

For an understanding of this disease, it is essential to get a clear conception of the clinical entity. It has been thought to be a disease of pregnancy, possibly of the nature of a toxæmia, but our work would suggest that this conception is false and that it is a condition occurring in the general female population, though more frequent in pregnant women. Major Sokley, in a personal communication, tells us that a similar condition is common among men in Bombay, if the two conditions are identical, which seems probable, then pregnancy must indeed be regarded merely as an exciting factor. It is the great severity and the dramatic course of the disease in pregnancy which has attracted the attention of medical workers and led to the entity being described as one of the toxæmias of pregnancy. But it appears rather that it is the extra demand made on the organism by the presence of the foetus that leads to an exacerbation of a disease already present. In this respect 'anaemia of pregnancy' resembles osteomalacia. In China, for example, osteomalacia occurs in both men and women, but because of the conditions of life, is far more common among women and particularly among pregnant women when the demand for calcium and phosphorus is enormously increased by the presence of the developing foetus.

The question of the identity of this anaemia and true pernicious anaemia must be considered. Pernicious anaemia has been defined by Cornell(9) as 'a disease of unknown ætiology, showing a characteristic triad of changes in the digestive, blood and nervous systems and progressing, usually by remissions, to a fatal termination'. On this definition it is obvious that the anaemia under discussion is not true pernicious anaemia. In the anaemia of pregnancy, digestive disturbances, diarrhoea, sore mouth, etc., do occur but the typical abnormality of this system—achlorhydria—recurring in practically 100 per cent of cases of true pernicious anaemia, is normally absent. This is a most significant diagnostic point

Whatever theories are held as to the ætiology of the two diseases, they must account for this striking and constant difference

Symptoms referable to the nervous system are also absent in the anæmia of pregnancy

The blood count and picture in both conditions are very similar, both show defective marrow activity and a similar response when regeneration takes place. But such blood findings are not diagnostic, they are only symptomatic, and in details, as for example the much more marked poikilocytosis and megaloblast formation in true pernicious anæmia, the two conditions differ. The feature of the blood which differs most strikingly, however, is the bilirubin content of the serum. In active pernicious anæmia the bilirubin content of the serum is typically raised, it being an index of the hæmolysis taking place. In 'pregnancy anæmia' the figures of the quantitative Van den Bergh reaction fall within normal limits, the average figure for the present series being 0.35 units, whereas Cornell(11) quotes results from cases of pernicious anæmia that give an average figure of 1.88 units. Such findings suggest that, whereas in true pernicious anæmia there is a hæmolytic agent responsible for the blood picture in the conditions under consideration, hæmolysis does not play an important part. In this respect and in the blood picture, this condition resembles the severe sprue anæmias reported by Mackie and his co-workers (12).

The evidence reviewed above differentiates this anæmia from true pernicious anæmia. The question then arises, is it due to some infective agent? As stated at the beginning of the paper, cases secondary to malaria, hookworm and syphilis have been excluded. The so-called idiopathic cases that remain have been thought by various workers, notably Acton (personal communication), to be due to a streptococcal infection from the gut. Others, on the analogy of pernicious anæmia, have considered a *B. welchii* infection to be responsible. The results of cultures of urine, fæces and resting gastric juice have lent no support to these views, neither have the animal experiments previously reported by Emmanuelov and Mehta (7). The streptococcal infections common in cases examined post-partum are probably of the nature of secondary infections, and the fact that many such cases occurred in groups suggestive of an epidemic of puerperal infection lends further support to this view. The presence of free hydrochloric acid in the gastric secretion of the majority of the cases makes an upward extension of the flora of the intestines unlikely. Many of the cases, however, do suggest a septic or infective condition as they may have high temperatures, either of typhoid (Widal negative in all cases) or septicæmic type. The fact that the temperature returns to normal with treatment by liver extract alone does not support an infective theory. Minot(13) has noted similar falls of temperature in cases of pernicious anæmia treated by liver extract and is of the opinion that the theory of an infective origin of that disease is no longer tenable.

The question of a toxæmia is more difficult. The recognized toxæmias such as eclampsia, pernicious vomiting and headaches are associated with both an

increase in blood pressure and alterations in the non-protein nitrogen constituents of the blood. The toxic state related to the high intestinal obstruction with supposed absorption of toxins from the gut, is characterized by high blood urea and low chloride values.

In the present series of cases the evidence, as far as it goes, is against the condition being a toxæmia of pregnancy as the blood pressure is low even in the severest cases and the few blood analyses available do not show the characteristic changes. In the most severe cases there is a slight increase in the non-protein nitrogen constituents, largely due to the raised urea values, but no more than could be expected in such a condition when the extreme anæmia would lead to some failure of kidney function. The chloride values show no reduction but on the contrary a rise, which is directly related to the degree of anæmia and therefore to the relative increase in plasma. The above evidence is, however, too scanty to be conclusive and further work is necessary before a toxæmia can be excluded.

If the evidence for an infective or toxæmia origin for this anæmia is at present unconvincing, is there any that would suggest a nutritional basis, other than the fact that liver extract is effective as a cure? The cure of the condition by liver extract is not in itself very convincing, as liver is active in any anæmia where the marrow function, though depressed, is still stimulatable and where there is enough iron present for hæmoglobin-formation. In the cases under consideration iron is not a limiting factor, as treatment by iron alone is ineffective whereas iron-free liver extract, without alteration of the diet or addition of iron, will effect a cure.

A study of the diets of these cases reveals defects which it is tempting to believe have some relation to the anæmia. The defects are lack of vitamins A and C, and in spite of the very varied diets eaten in the different communities studied these defects are constantly present. In certain cases, among Mahomedans, a large amount of ghee is eaten, which might be thought to be approaching an adequate amount. But the fact that in such households very severe cases of rickets and mild cases of osteomalacia occur suggests that the ghee is either adulterated with vegetable oils or else, by its method of preparation, deprived of its vitamins. Such ghee in households that do not observe purdah, and in Bombay only Mahomedans do, would not necessarily give rise to signs of a D deficiency, as the members of such households would be exposed to sunlight, which would compensate for the D but not for the A deficiency. The frequent finding of low serum calcium and blood inorganic phosphate values in the non-purdah cases, however, suggests that there is even in these cases an associated mild D deficiency, pointing to an inadequate fat intake.

It could be argued that the whole of the female section of the poorer classes in Bombay is suffering from a similar deficiency, but the general prevalence of a mild anæmia, 3 million odd cells being an average red count for many so-called normal pregnant women, only gives further support to a deficiency theory of the causation of this disease. That the extra strain of pregnancy should precipitate



symptoms is easily understandable. It is not thought that there is an absolute deficiency in either vitamin, for if there were, xerophthalmia and scurvy would occur, but that there is a relative deficiency made worse when the extra demands of the foetus are added to the normal maternal metabolism.

The animal experiments reported are very suggestive. A diet deficient in the same elements as those that are lacking in the diets studied in Bombay produces an extreme and fatal anæmia, the majority of the animals affected being pregnant though a similar condition occurred less frequently and generally in a less severe form in non-pregnant animals (9). Koessler and others (14) have reported a similar anæmic state in rabbits on a vitamin A deficient diet. Our investigations up to the present time lead us to believe, therefore, that it is along nutritional lines of investigation that the causation of 'pregnancy anæmia' will be found. We propose, therefore, to put to the test of animal experimentation our working hypothesis that 'the fundamental factor in the causation of the anæmias of pregnancy is chronic insufficiency of vitamins A and C'.

Our thanks are due to Dr Margaret Balfour for much help and advice, and to the Staff of the Cama and Wadia Hospitals, Bombay, for permission to work on their cases, also to Mrs Talpade, M B, for her assistance in the wards and the laboratory.

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for treatment

Condition	Red h	BLOOD PICTURE	Punctate leucocytes	Hemoglobin
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AVERAGE				1,320,000	20	0.7	4,600	+	-
14	Do	10	1,710,000	27	0.78	3,800	+	-	-
30	Do	11	2,350,000	35	0.80	11,600	-	-	-
54	Do	13	1,220,000	25	1.0	9,400	++	-	-
61	Do	33	2,150,000	35	0.8	4,600	+	-	-
			1,645,000	26.2	0.93	6,317			

course lasted for a total of 28 days. The results, as compared with the treatment of a similar type of patient given the cinchona alkaloids, were exceptionally good.

Some of the conclusions reached were that 'plasmoquine was much more effective in producing a permanent cure in benign tertian malaria' than was quinine, but that 'the margin of safety in the dosage of plasmoquine renders it necessary that further experiments should be carried out to determine the best dosage and duration of treatment, before the drug is issued for general use outside hospitals'. The combination of quinine with plasmoquine seemed to be more effective in the production of both clinical and radical cures of the disease than plasmoquine alone.

Since the completion of the research previously recorded, further work has been carried out with the object of obtaining more definite information on the optimum dosage of the drug and the minimal duration of treatment necessary to produce a permanent cure of the disease. The results of the continuous course of treatment with plasmoquine and quinine combined, made it seem probable that a high percentage of radical cures might be obtained by continuous treatment with smaller doses of plasmoquine, if combined with the same doses of quinine as before, and at the same time the toxic manifestations might be reduced, if not entirely abolished, by such treatment. Even if a smaller dosage was followed by a few relapses it would be of minor importance, if the toxic symptoms could be avoided, because it would open up the benefits of such treatment to patients who could not be kept under strict observation and hospital control.

#### DETAILS OF THE METHODS, ETC., USED IN THE RESEARCHES

The routine procedure used in testing the properties of plasmoquine was similar to that already given in detail in previous articles of this series. The patients were all young European soldiers from the same population as that described in previous work, and were treated under similar conditions (Sinton, 1927). The patients were sent to the Treatment Centre because treatment with the cinchona alkaloids had failed to produce a radical cure of the disease after many attempts.

These patients on arrival at Kasauli were subjected to weekly blood examinations by the thick-film method and in no case was specific treatment begun, unless *P. vivax* was detected in the peripheral blood immediately before the commencement of treatment. As in previous researches, a preliminary purgation with calomel and magnesium sulphate was given before any course of specific treatment was started.

#### TREATMENT WITH PLASMOQUINE AND QUININE BY THE ORAL ROUTE

The combination of plasmoquine and quinine, 'plasmoquine compound,' issued by the makers, contains 0.125 grm (2 grains) of quinine to every 0.01 grm of

plasmoquine\* For reasons which will be discussed later, it was decided not to use 'plasmoquine compound' as such, but to give tablets of pure plasmoquine, followed by quinine sulphate in solution in two daily doses of 0.625 gm (10 grains) each, the mixture used being the quinine-sulphate-citric-acid-magnesium-sulphate one described in previous work (Sinton, 1926a)

Two series of patients were started on this type of treatment for a period of 21 days continuously, with as few intervals as the development of toxic symptoms would allow. The daily dosage in Series I was 0.06 gm plasmoquine and in Series II 0.04 gm, but as will be seen later the occurrence of many toxic effects prevented some of the patients in Series I from completing the full course of 21 days with plasmoquine.

*Series I* The scheme mapped out for this series was to give 0.06 gm plasmoquine daily, supplemented by a daily dosage of 1.25 gm (20 grains) quinine sulphate in solution. The drugs were given in doses of half these amounts twice daily. The treatments were started with some of the original consignment of plasmoquine supplied free by the makers, but when this was exhausted at the end of June 1928, a new consignment purchased in India in March 1928 was commenced. Within a few days after the commencement of the new supply of the drug severe toxic symptoms occurred in several cases, so plasmoquine treatment was suspended in this series. In those cases whose plasmoquine treatment had not been completed, the daily dose of 1.25 gm (20 grains) of quinine was continued until the 21st day of treatment was finished.

Seventeen patients were started on this line of treatment, of whom 7 completed the full 21 days' course of plasmoquine and quinine, six patients completed 8, 9, 11, 14, 16 and 17 days respectively with plasmoquine and quinine and the remaining four received only 4 days of the combined treatment. All the patients completed the full course of 21 days of quinine treatment.

*Series II* On account of the sudden development of many toxic symptoms in the cases mentioned above, it was decided that, with this sample of the drug, doses of 0.06 gm daily were too great, so a new series of treatments was commenced in which the daily dose of plasmoquine was 0.04 gm and that of quinine the same as before, i.e., 1.25 gm (20 grains) daily. This course was carried out for 21 days.

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\*The original 'plasmoquine compound' issued, contained 0.01 gm plasmoquine and 0.125 gm quinine sulphate combined in tablet form. Since then the makers have issued another tablet containing only half these amounts of the drugs. This has led to some confusion when treatments are recorded in the number of pills given daily, as has been done in several reports on this form of treatment. We also understand that in India, where this drug can be bought without a medical prescription, patients have been known to take, not realizing the difference, the larger tablets instead of the smaller ones, and in the same numbers, and that dispensers have made similar mistakes. The necessity for an accurate statement of dosage cannot be too strongly emphasized. On two occasions we have noticed in annotations and abstracts of work on plasmoquine treatment that, through a shift of the decimal point, the dose recommended was ten times that given in the original, indeed in one such annotation the daily dose of the drug is given as 2 grms!!!

TREATMENT WITH INTRAMUSCULAR INJECTIONS OF PLASMOQUINE COMBINED  
WITH QUININE GIVEN ORALLY

Through the kindness of the Havero Trading Co, Calcutta, the agents of the I G Farbenindustrie Aktiengesellschaft, who manufacture plasmoquine, we were supplied with samples of a 1 per cent aqueous solution of plasmoquine in capsules for use by intramuscular injection. The makers recommend this method of treatment 'in cases of tertian and quartan malaria which run a severe course accompanied by mental torpor or coma, further in cases where intestinal disturbances preclude effective oral administration and in blackwater fever at all stages'.

The instructions for injection were as follows — 'The aqueous solution is generally injected intramuscularly, but the intravenous route can also be used. The outer and upper quadrant of the gluteal region is the most satisfactory site of injection. The injections are well borne and do not give rise to disturbing sequelæ or prolonged pain, nor do they cause infiltrations. Subcutaneous injection should be avoided, as it is followed by a burning sensation at the site of injection which lasts for some hours, although it does not cause inflammation'.

The dosage recommended in tertian and quartan malaria was a daily injection of 3 to 6 c.c. of the solution (i.e., 0.03 to 0.06 gm. plasmoquine) for one week, followed by a rest of 4 days, then the treatment is resumed in the same dosage for 3 days followed by a second interval of 4 days. This after-treatment of 3 days' treatment and 4 days' interval is to be continued for 5 weeks.

From our experience of the benefits of a combined quinine and plasmoquine treatment, it was decided to try small intramuscular injections of plasmoquine and at the same time to give quinine solution by the mouth.

*Series III* Six patients were treated with intramuscular injections of 0.03 gm. plasmoquine daily into the gluteal region for 6 days and at the same time 10 grams of quinine in solution was given daily by the mouth. After the injections were finished, the quinine dosage was increased to 20 grams in two daily doses of 10 grams each continued for a further week. The injections were well borne and no pain was complained of at the site of injection nor was any inflammation noted. No toxic symptoms were observed in this series.

RESULTS OF TREATMENT IN THE PREVENTION OF RELAPSE

Relapses were diagnosed as heretofore by the finding of parasites in the peripheral blood by the thick-film method of examination. These examinations were carried out weekly for at least eight weeks after the completion of all treatment.

The results of treatment are summarized in Table I in which have also been included, for the purpose of comparison, the results of our previous work with plasmoquine and plasmoquine compound (Sinton and Bird, 1928).

*Series I* The seventeen patients in this series completed their full period of observation after treatment and in no instance was a relapse observed. These results go to show that even smaller doses of plasmoquine than those originally

TABLE I

Series	TREATMENT GIVEN					Total cases	Cases not relapsing but observed less than 8 weeks	Failures observed	PERCENTAGE OF FAILURES				
	DAILY DOSE X DAYS		TOTAL DRUG GIVEN						Observed	Possible maximum	Observed minimum	Average	
	Plasm	Quinine	Grammes	Plasm	Quinine								Grams
PM 1 *	0.08 X 17	Nil	Nil	1.36	Nil	29	1	10	35.7	38.0	34.5	36.0	
PM 2 *	0.08 X 28	Nil	Nil	2.24	Nil	22	0	5	22.7	22.7	22.7	22.7	
PMC 1 *	0.10 X 17	20 X 17	340	1.70	340	15	0	3	20.0	20.0	20.0	20.0	
PMC 2 *	0.10 X 28	20 X 28	560	2.80	560	20	2	0	0.0	10.0	0.0	3.4	
PMQ I	0.06 X 4 — 0.06 X 21	20 X 21	420	0.24—1.26	420	17	0	0	0.0	0.0	0.0	0.0	
PMQ II	0.04 X 21	20 X 21	420	0.84	420	44	2‡	3	7.1	11.3	7.0	8.4	
PMQ III	0.03 X 6†	10 X 6 20 X 7	200	0.18	200	6	1	0	0.0	16.6	0.0	5.9	
Controls	Nil	30 X 21	630	Nil	630	36	0	15	41.6	41.6	41.6	41.6	

\* Series recorded in previous work (Sinton and Bird, 1928)

† Intramuscular injection

‡ Later clinical history records no relapses

suggested are capable of producing a permanent cure in a large number of cases of chronic benign tertian malaria. The fact that some of these patients only received 4 days of plasmoquine treatment, suggests that shorter courses of plasmoquine, if followed by more prolonged quinine treatment, may also be an efficient method of treatment, or least deserves further trial.

*Series II* Amongst the 44 patients in this series relapses were detected in three cases but in two patients a full observation period of 8 weeks by blood examination could not be carried out, the observation periods being 2 and 5 weeks respectively. The later histories of these two cases were traced and in neither instance had relapse been reported at a later date.

From these figures it would seem that a high percentage of permanent cures can be obtained, with little toxic risk in a robust population, with even as small a daily dosage as 0.04 grm of plasmoquine, if this is combined with quinine. The percentage is so high that it seems to us doubtful whether a higher dosage is justifiable in an attempt to obtain a few extra cures, which could probably be obtained by another course of treatment if relapse occurs.

*Series III* One of the six patients treated by intramuscular injection was lost sight of before the end of the period of observation and in none of the other five was a relapse detected. The number of patients treated with this low dosage of plasmoquine are too few on which to generalize, but suggest that even smaller doses than 0.04 grm of plasmoquine, in combination with quinine, may produce a high percentage of permanent cures.

*Quinine Control Series* Among the 38 controls, relapses were observed in 15 or about 42 per cent.

#### THE EFFECTS OF TREATMENT ON THE DURATION OF *P. vivax* IN THE PERIPHERAL BLOOD

For the purposes of comparison the duration of parasites in the peripheral blood as determined by the thick-film method have been given in Table II, in which the results observed in our previous work (Sinton and Bird, 1928) have been included.

From this table it will be seen that in only two instances (3 per cent), out of the 71 patients observed, were parasites found after 36 hours from the commencement of treatment, when quinine was given in solution along with plasmoquine, while in previous work where the quinine was given in tablet form in the same doses and with larger doses of plasmoquine, 17 per cent of the cases still showed parasites after 48 hours. The quicker rate of disappearance in the former case may have been due to the better absorption of the quinine from the standard quinine solution than from the tablets.

From these results it would appear that the combination of plasmoquine and quinine has probably a more rapid action in clearing the peripheral blood of parasites than either drug given alone.

TABLE II

Series	TREATMENT			Total cases	NUMBER OF CASES SHOWING PERCENT AFTER HOURS —															
	Drugs	DAILY DOSES			Cases	Per cent	0		24		36		48		72		96		120	
		Plasm	Quinine				Cases	Per cent	Cases	Per cent	Cases	Per cent	Cases	Per cent	Cases	Per cent	Cases	Per cent		
Grammes	Grains																			
PM *	Plasm	0.08	Nil	46	46	100	42	91.0	?	?	26	56.5	8	17.4	3	6.5	0	0	0	0
PMC *	Pl Comp	0.10	20	34	34	100	18	53.0	?	?	6	17.0	1	3.0	0	0.0	0	0	0	0
PMQ I	Pl and Q	0.06	20	17	17	100	8	47.0	0	0	0	0.0	0	0.0	0	0.0	0	0	0	0
PMQ II	Pl and Q	0.04	20	48	48	100	13	27.1	2	4.1	0	0.0	0	0.0	0	0.0	0	0	0	0
PMQ III	Pl and Q	0.03†	10	6	6	100	5	83.3	0	0	0	0.0	0	0.0	0	0.0	0	0	0	0
Control	Quinine	Nil	30	36	36	100	18	50.0	4	11.1	1	3.7	0	0.0	0	0.0	0	0	0	0

\* The plasmoquine and plasmoquine compound series already recorded (Sinton and Bird 1928)

† By intramuscular injection



APPENDIX II—*concl'd*

Months	Total children examined	Children with enlarged spleen	Spleen-rate	Average spleen	Average enlarged spleen
1928					
May	250	219	87.6	3.39	3.72
June	193	162	83.9	3.00	3.37
July	192	161	83.9	3.02	3.42
August	207	176	85.0	3.24	3.60
September	218	191	87.6	3.32	3.64
October	231	214	92.6	3.56	3.77
November	226	211	93.4	3.73	3.92
December	234	217	92.7	3.96	4.19

APPENDIX III

*Simultala Spleen Records, 1926—1928*

Months	Total children examined	Children with enlarged spleen	Spleen-rate	Average spleen	Average enlarged spleen
1926					
June	91	61	67.0	2.73	3.59
July	89	63	70.8	2.81	3.55
August	89	67	75.3	2.89	3.52
September	89	72	80.9	3.08	3.58
October	92	77	83.7	2.98	3.36
November	81	66	81.5	3.95	3.52
December	89	71	79.8	2.87	3.35
1927					
January	87	70	80.5	2.49	3.28
February	83	57	68.7	2.41	3.05
March	87	59	67.8	2.38	3.03
April	89	60	67.4	2.34	2.98
May	84	51	60.7	2.05	2.74
June	87	48	55.2	1.88	2.60

## Seasonal Variations of the Spleen-Rate

APPENDIX III—*concl'd*

Months	Total children examined	Children with enlarged spleen	Spleen-rate	Average spleen	Average enlarged spleen
1927					
July	87	51	58.6	1.94	2.60
August	86	52	60.5	1.95	2.57
September	86	58	67.4	1.95	2.41
October	86	68	79.1	2.48	2.88
November	90	75	83.3	2.94	3.33
December	85	69	81.2	2.98	3.45
1928					
January	76	68	89.5	3.27	3.54
February	74	65	87.8	3.17	3.47
March	76	67	88.2	3.04	3.31
April	77	63	81.8	2.91	3.33
May	77	61	79.2	2.75	3.21
June	65	42	64.6	2.22	2.88
July	62	41	66.1	2.24	2.87
August	65	44	67.7	2.53	2.93
September	68	49	72.1	2.43	2.98
October	73	60	82.2	2.62	2.96
November	71	58	81.7	2.93	3.36
December	72	59	81.9	3.28	3.78

## APPENDIX IV

## Harishpur Spleen Records, 1926—1928

Months	Total children examined	Children with enlarged spleen	Spleen-rate	Average spleen	Average enlarged spleen
1926					
June	118	107	90.7	4.05	4.36
July	116	105	90.5	3.80	4.09
August	112	104	92.9	3.94	4.17
September	116	110	94.8	4.06	4.22

APPENDIX IV—*concl'd*

Months	Total children examined	Children with enlarged spleen	Spleen-rate	Average spleen	Average enlarged spleen
1926					
October	119	116	97.5	4.04	4.12
November	110	107	97.3	3.95	4.03
December	113	110	97.3	3.65	3.72
1927					
January	109	103	94.5	3.47	3.62
February	109	101	92.7	3.20	3.37
March	103	94	91.3	3.15	3.35
April	107	97	90.7	3.07	3.28
May	112	93	83.0	2.82	3.19
June	111	85	76.6	2.53	3.00
July	111	86	77.5	2.55	3.01
August	109	83	76.1	2.35	2.78
September	117	92	78.6	2.25	2.57
October	125	113	90.4	3.21	3.45
November	125	113	90.4	3.44	3.71
December	124	113	91.1	3.57	3.82
1928					
January	121	114	94.2	3.58	3.74
February	121	112	92.6	3.52	3.72
March	117	106	90.6	3.24	3.48
April	116	105	90.5	3.13	3.36
May	116	101	87.1	3.03	3.32
June	88	80	90.9	2.93	3.12
July	96	86	89.6	3.03	3.26
August	98	88	89.8	3.12	3.36
September	107	99	92.5	3.28	3.47
October	111	107	96.4	3.55	3.64
November	108	106	98.2	3.65	3.70
December	111	107	96.4	3.78	3.88

## Seasonal Variations of the Spleen-Rate

## APPENDIX V

## Jatrapur Spleen Records, 1926—1928

Months	Total children examined	Children with enlarged spleen	Normal spleen	Spleen-rate	DETAILS OF SPLENIC ENLARGEMENT						Average spleen	Average enlarged spleen
					F1	F2	F3	F4	U	BU		
1926												
June	66	63	3	95.5	13	13	11	12	6	8	4.00	4.15
July	65	63	2	95.9	11	21	12	4	8	7	3.88	3.97
August	61	60	1	98.4	8	16	17	2	7	10	4.18	4.23
September	65	65	0	100.0	4	14	23	7	7	10	4.45	4.45
October	65	65	0	100.0	0	9	25	12	11	8	4.75	4.75
November	56	56	0	100.0	0	7	18	14	9	8	4.87	4.87
December	59	59	0	100.0	0	1	13	28	12	5	5.12	5.12
1927												
January	59	59	0	100.0	0	1	17	24	10	7	5.08	5.08
February	59	59	0	100.0	0	1	13	25	9	11	5.27	5.27
March	50	50	0	100.0	0	5	17	15	9	4	4.80	4.80
April	53	53	0	100.0	0	5	19	18	8	3	4.72	4.72
May	50	50	0	100.0	1	5	23	13	6	2	4.48	4.48
June	56	56	0	100.0	1	8	28	14	5	0	4.25	4.25
July	53	53	0	100.0	1	8	26	14	4	0	4.23	4.23
August	54	54	0	100.0	2	14	23	10	5	0	4.04	4.04
September	53	53	0	100.0	9	20	12	7	5	0	3.60	3.60
October	53	53	0	100.0	1	19	20	9	4	0	3.92	3.92
November	57	57	0	100.0	0	2	10	24	16	5	5.21	5.21
December	54	54	0	100.0	0	0	7	25	17	5	5.37	5.37
1928.												
January	59	59	0	100.0	0	0	8	24	16	11	5.51	5.51
February	56	56	0	100.0	0	1	10	21	15	9	5.37	5.37
March	54	54	0	100.0	0	2	9	21	13	9	5.33	5.33
April	57	57	0	100.0	0	3	10	22	13	9	5.26	5.26
May	57	57	0	100.0	0	5	14	19	15	4	5.00	5.00
June	40	40	0	100.0	8	7	4	8	8	5	4.40	4.40
July	34	3	0	100.0	5	8	3	6	7	5	4.50	4.50
August	44	44	0	100.0	3	6	11	8	10	6	4.77	4.77
September	43	43	0	100.0	3	4	11	11	9	5	4.80	4.80
October	47	47	0	100.0	2	5	9	11	12	8	5.06	5.06
November	47	47	0	100.0	1	5	10	10	13	8	5.13	5.13
December	51	51	0	100.0	0	3	11	14	12	11	5.33	5.33

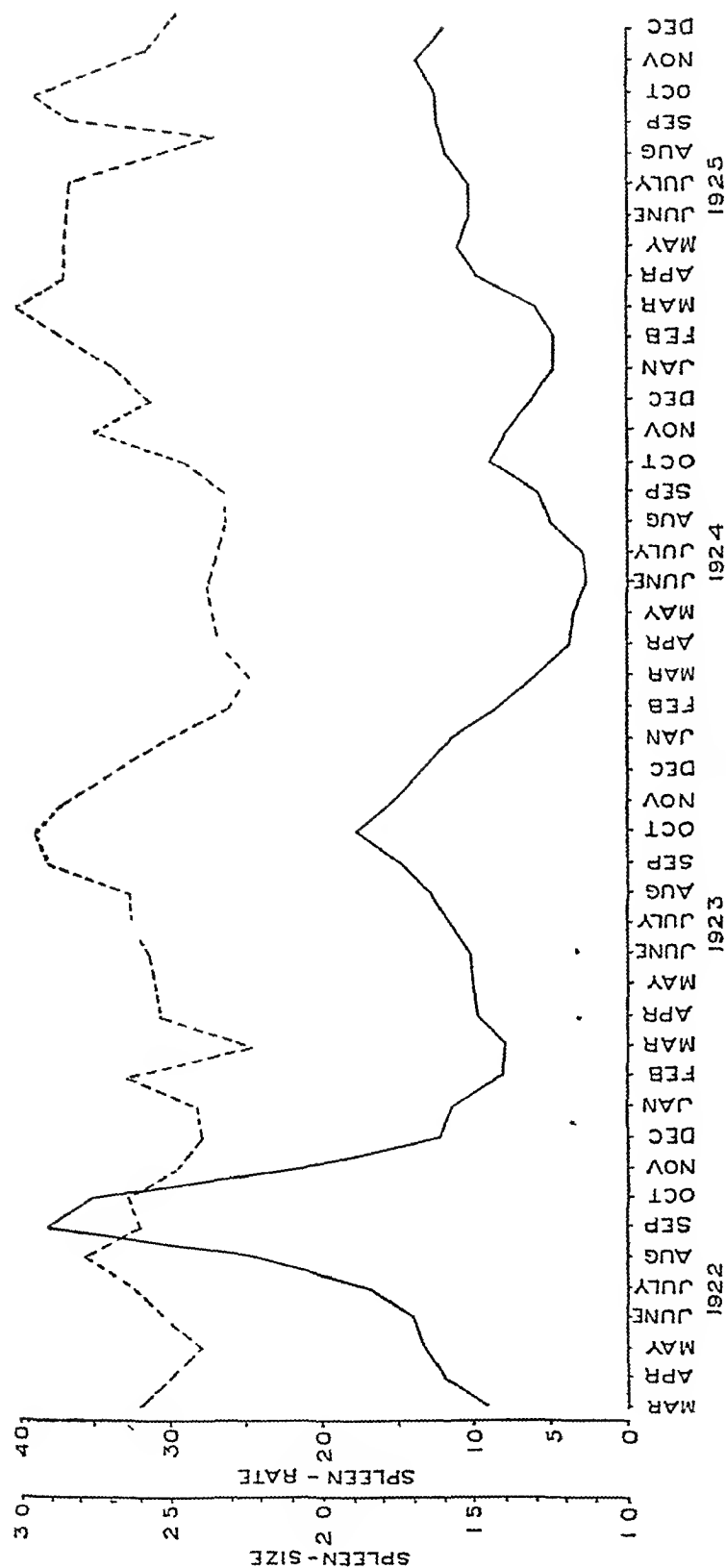


CHART 1  
SONARPUR VILLAGES  
*Monthly variations in spleen-rate and spleen size*

References —  
Spleen-rate  
Average spleen  
Average enlarged spleen

CHART 2  
KRISHNANAGAR VILLAGES  
*Monthly variations of spleen index and average spleen*

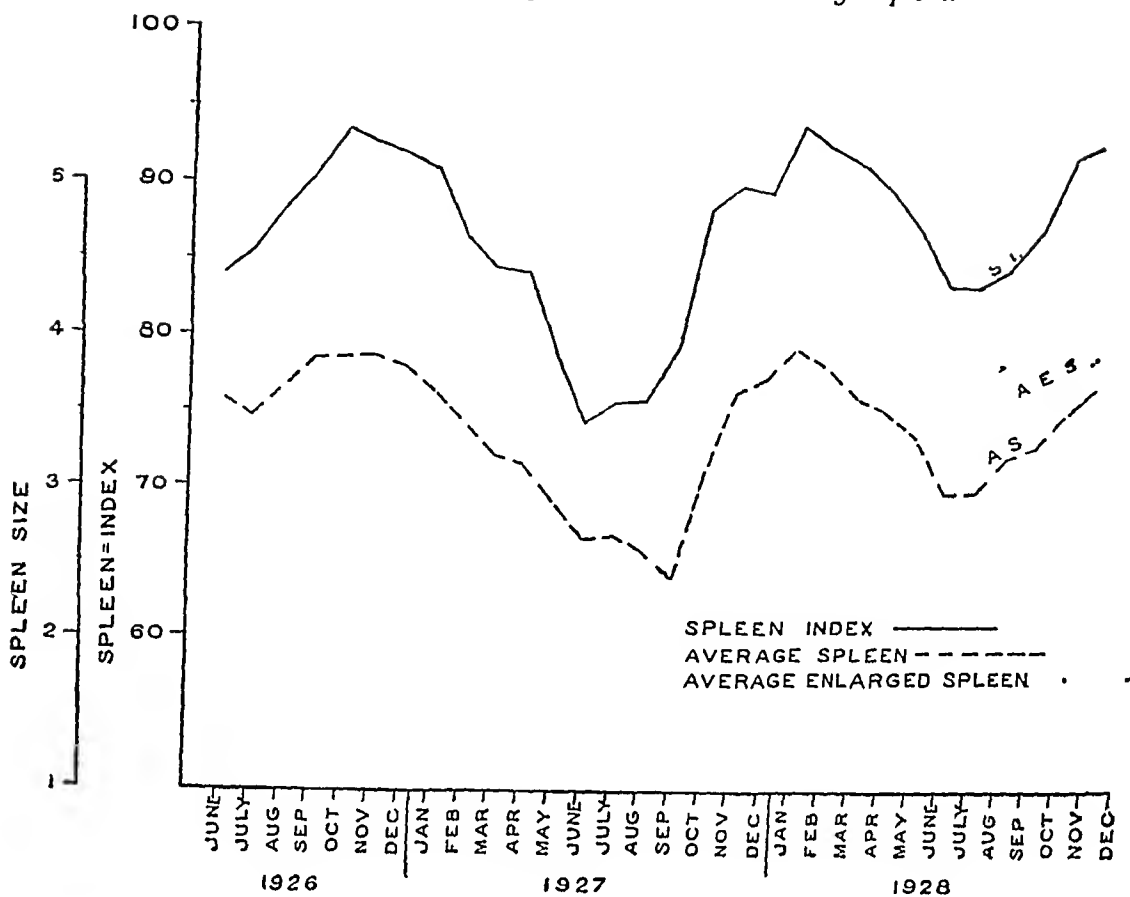


CHART 3

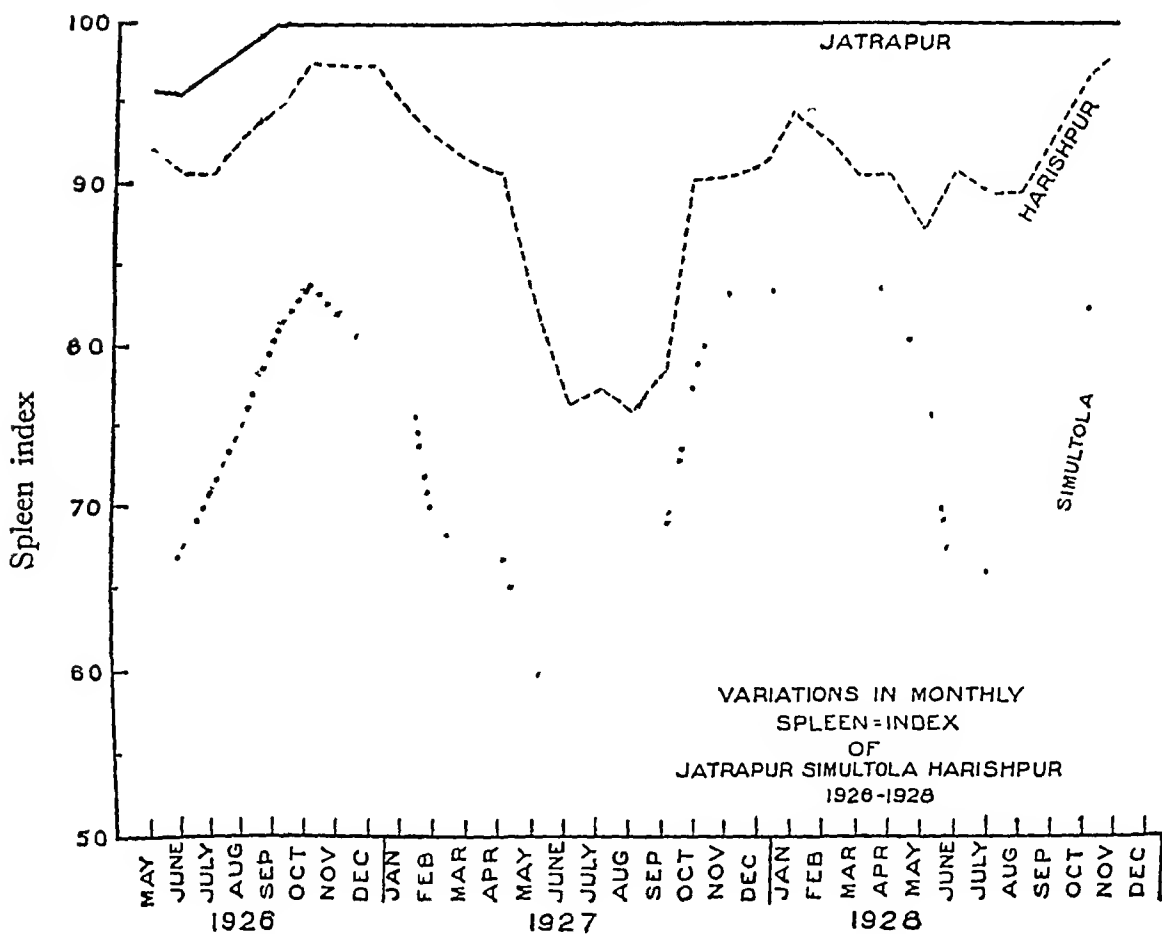


CHART 4

SIMULTALA

Monthly variations in spleen index, average spleen, and average enlarged spleen, 1926—1928

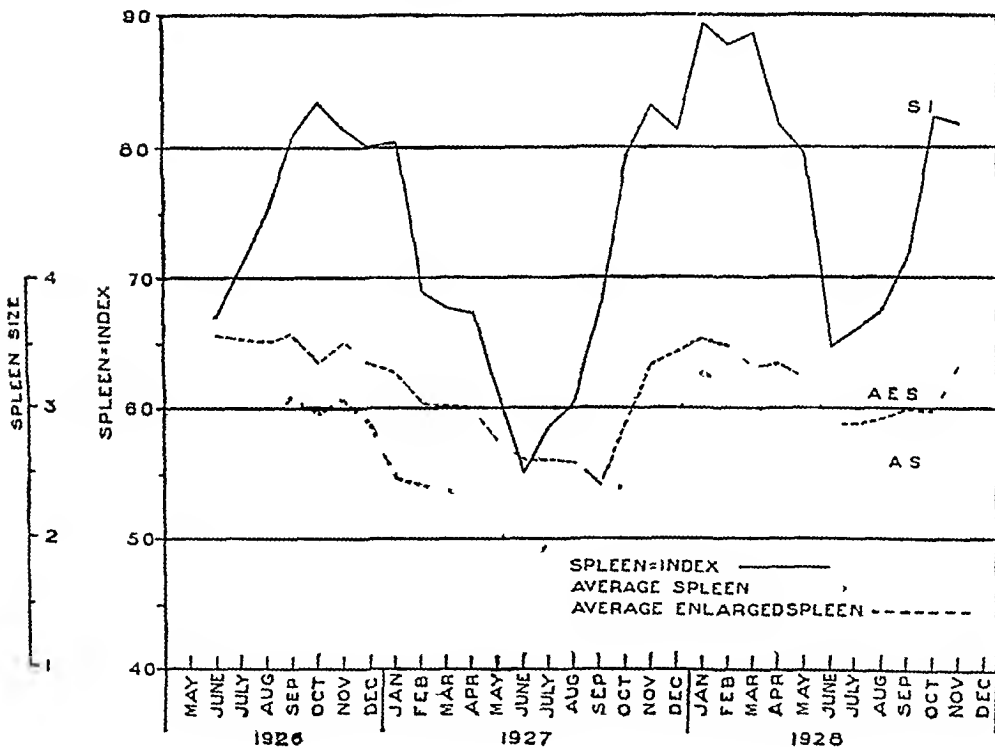


CHART 5  
HARISHPUR  
*Monthly variations of spleen index and spleen size*

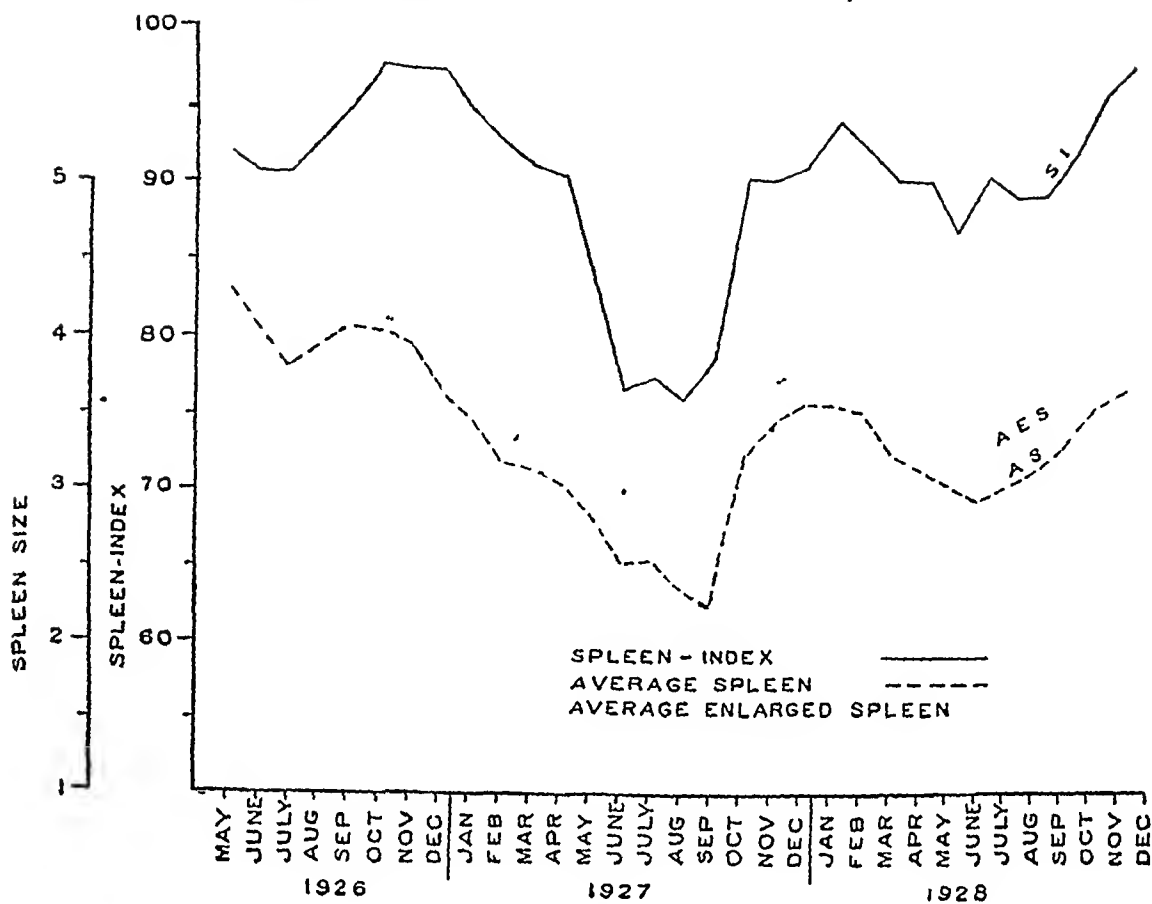
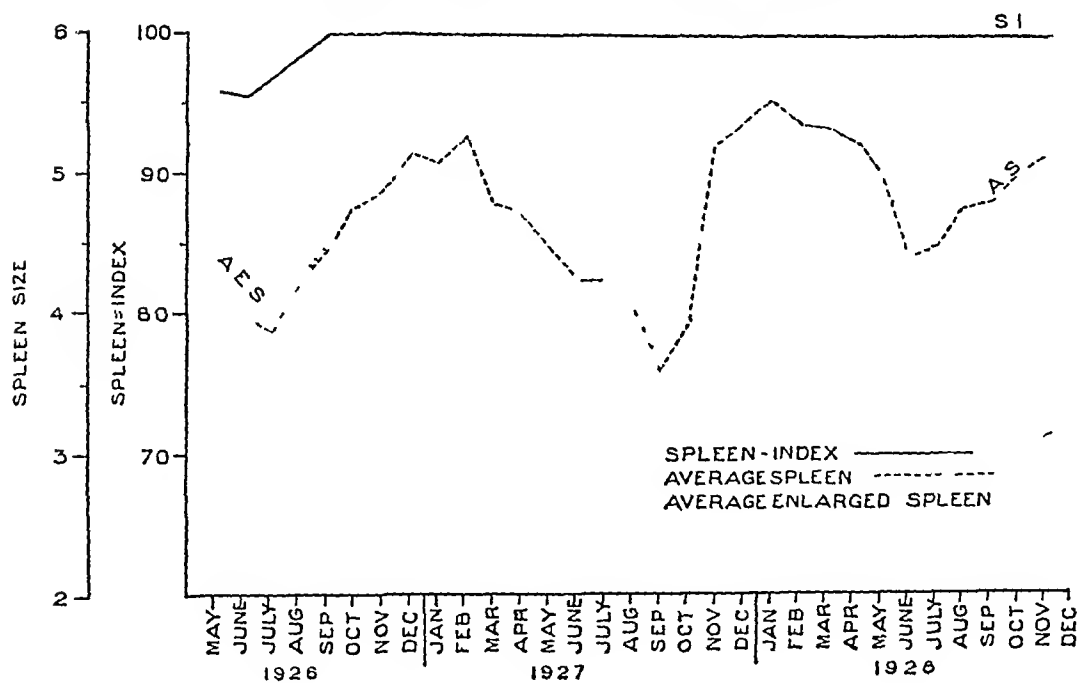


CHART 6  
JATRAPUR  
*Monthly spleen index and average spleen*





# THE DEPIGMENTED PATCH IN LEPROSY A CLINICAL AND PATHOLOGICAL STUDY

BY

JOHN M HENDERSON, M B, Ch B (Glas),

*Working under the British Empire Leprosy Relief Association at the School of Tropical Medicine and Hygiene, Calcutta*

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## (1) THE RELATIONSHIP OF THE DEPIGMENTED PATCH OF LEPROSY TO THE DISEASE AS A WHOLE

INABILITY to produce a condition of generalized leprosy in lower animals, together with the prolonged incubation period of the naturally-occurring disease in man, prevent the definite statement that the depigmented patch represents the initial lesion in all cases of leprosy, or even in a majority of cases. The frequency with which depigmented patches make their appearance as first-noticed lesions has, however, been noted by various workers. Rogers and Muir (1925), in an analysis of first-noticed lesions in 252 Indian patients, found that depigmented patches constituted the apparent primary lesion in 212 (84.1 per cent). Gomez, Basa, and Nicolas (1922), in their studies of early lesions in the children of lepers in the Philippine Islands, described the appearance of whitish patches resembling morphaea spots as the earliest manifestation of the disease in a considerable proportion of their cases. This feature has also been noted in the children of lepers in India. On the other hand, there is one group of cases in which the depigmented patch is almost certainly not the primary lesion, namely, those patients in whom the onset of the disease is signalized by the simultaneous appearance of multiple depigmented patches in different parts of the body. Such cases represent an active dissemination of the disease from a latent focus either in the lymphatic glands or possibly in the nasal mucous membrane.

## (2) CLINICAL TYPES OF THE DEPIGMENTED PATCH

Several varieties of depigmented patches can be observed clinically.

(a) The first variety—which we may describe as the peri-follicular type—takes the form of a collection of discrete pin-head-like spots occurring around

the mouths of the hair follicles. In addition to the depigmentation there is a mild degree of hyper-keratosis around the mouths of the hair follicles and a distinct sensation of roughness is conveyed to the palpating finger. This peri-follicular type of lesion is, in our experience, of recent occurrence that shown in the coloured Plate II, fig 1 was, according to the statement of the patient, an intelligent woman, only of 15 to 20 days' duration.

(b) The second variety—which may be regarded as a further development of the peri-follicular type—presents itself as a more or less uniformly depigmented area of varying size. The edge of the patch may shade off into apparently normal skin or may present a peri-follicular zone of depigmentation. The latter appearance is indicative of a progressive lesion where it occurs in a patch which has hitherto presented a uniformly depigmented appearance, it is indicative of a re-activation of the lesion. Broken stunted hairs can be detected throughout this type of lesion and variable degrees of keratosis are commonly present (Plate III, fig 2). Where a single depigmented patch is present on the skin surface, this is the type most commonly met with. The duration of such patches varies considerably in different cases, but they are commonly of much longer standing than the peri-follicular type. Those which have existed for years, showing no tendency either to extend or to regress, are essentially of the nature of scars.

(c) The third variety is essentially an extension of the simple flat depigmented patch described above. It takes the form of a large plaque-like lesion covering a considerable area of a limb, usually the extensor aspect, or a large portion of the trunk. Well-marked keratosis, amounting in some cases to a condition similar to ichthyosis, is frequently met with in this type of lesion. Lesions of this type are very chronic, that shown in Plate III, fig 3 is of at least 20 years' duration.

(d) The 'zone' type of patch represents a transition stage between the true depigmented patch and the erythematous lesion. The appearance presented is that of a flat depigmented patch bounded, either wholly or partially, by a raised erythematous border (Plate IV, fig 4).

(e) The 'mottled' type of patch is one which is not very commonly seen in untreated cases. It presents the appearance of a depigmented patch in which there is a return of pigment around the mouths of the hair-follicles. This type of lesion is much more commonly observed in cases undergoing treatment and arises as the result of the application of counter-irritants to a patch in which depigmentation was previously more or less uniform (Plate IV, fig 5).

### (3) ATTRIBUTES OF THE DEPIGMENTED PATCH

These have been very fully dealt with by other workers and need only be touched on here.

(a) *Depigmentation*—This varies in degree, tending naturally to be more striking in dark-skinned patients. Excluding this, however, variations in the degree of depigmentation occur in patients whose skins are of approximately equal tint. In general, recent patches show relatively more depigmentation than those







Fig 2—Uniformly depigmented patch showing broken stunted hairs



Fig 3—'Plaque' type of depigmented patch showing marked keratosis



of longer standing. It would appear that there is a partial restoration of the pigment-forming function of the skin in certain quiescent and long standing cases, even in the absence of treatment. Lesions of the palms and soles show relatively a less striking loss of pigment than those elsewhere, owing to the fact that the natural deposition of pigment in these areas is less in amount than on the body surface generally. Depigmentation in leprosy is relative, not absolute, and is very seldom comparable in degree to that seen in leucoderma.

(b) *Anæsthesia to light touch* This is a variable feature. It is very commonly present in the simple flat depigmented patch and in the plaque type of lesion, paræsthesia is, in our experience, a more common finding than superficial anæsthesia in the peri-follicular type of lesion. In the 'zone' type of lesion, anæsthesia at the centre with paræsthesia at the periphery is the common finding. In the relatively few examples of the 'mottled' type of lesion that we have been able to examine, anæsthesia has been present. In these cases treatment was apparently of some value in stimulating recovery of the pigment-forming function of the skin only. In general, lesions of the limbs are more commonly anæsthetic than those of the trunk. (The papers of Monrad-Krohn, Rogers and Muir, and Rodriguez, should be consulted for a more extensive review of this aspect of the subject.)

(c) Para- and hyper-keratosis and distortion of the hair follicles have already been noted. Anhydrosis is also commonly present in depigmented patches and there may be compensatory hyperidrosis in the immediate neighbourhood.

#### (4) HISTOLOGICAL APPEARANCES

The histological appearances vary with the type of lesion, in the earliest type, the predominant feature is a proliferation of endothelial (or endothelial-like) cells, confined at first to the central area of the papillæ of the corium, along the hair follicles, surrounding the sweat and sebaceous glands, and along the lines of the subpapillary and corial lymphatic plexuses (Plates V, fig 6 and VI, fig 7). In slightly more advanced types of lesions one finds that the proliferation of cells is not confined to the areas mentioned above, but that it fills up the papillæ of the corium, forms a definite mantle around the hair follicles, sweat and sebaceous glands, and invades the portions of the corium adjacent to the subpapillary and corial lymphatic plexuses (Plate VI, fig 8). Another feature of interest in the depigmented type of lesion is the increase in numbers of the so-called 'mast' cells. These cells are present in normal connective tissue; they show up well when stained by Ziehl-Neelsen's method and present the appearance of small, irregularly oval or spindle shaped structures containing dark red or purplish granules. In a considerable proportion of the depigmented patches that we have examined there is a definite increase in the numbers of these cells.

In patches in which the depigmentation is a well marked clinical feature, sections stained by Levaditi's silver method show a diminution in the concentration of melanin pigment granules, particularly in the basal-cell layer of the epithelium.

(Plate VII, figs 9 and 10) The appearances are naturally not so striking as in leucoderma

In the simple and relatively recent depigmented patch there is little apparent reaction on the part of the tissues to the invasive process. Lymphocytes can be seen among the proliferating endothelial-like cells but fibroblasts do not constitute a prominent feature. In the chronic quiescent type of patch, the appearances are essentially those of scar formation with fibrosis and sclerosis along the lines of previous cellular proliferation (Plate V, fig 11)

The 'zone' type of lesion, which appears to represent a transition stage between the depigmented patch and the erythematous type of lesion, shows rather a different picture microscopically. Endothelial cell proliferation and tissue reaction are both more intense than in the simple flat depigmented patch and there is dilatation and apparent new formation of capillary blood vessels. Fibroblast and lymphocyte proliferation break up the accumulation of endothelial-like cells into more or less isolated foci, giant cells are commonly present, and the whole picture closely resembles a tubercle follicle with the notable exception that necrosis is not present—at least in our experience (Plate VII, fig 12)

These appearances have been described in a previous paper (Henderson, 1928)

The relationship of the specific organism of the disease, *Mycobacterium lepræ*, to the depigmented patch is a very variable one. Organisms are always few in number and in patches of long standing none may be found even after prolonged examination of serial sections

Where organisms are present they may be either inside the endothelial-like cells, or at other times, inside the lumina of lymphatic vessels, or again, free in the surrounding tissues. Where the organisms are intra-cellular, the cells containing them manifest no degenerative changes. This feature combined with the lack of tissue response—at least in the simple depigmented patch—indicates the essentially low pathogenicity of the organism at this stage of the disease

The acid-fast granules of 'mast' cells, which in addition to being inside the cells may also be extruded into the surrounding tissues, must be differentiated. Aggregations of such granules may closely resemble aberrant forms of *M lepræ*. Acid-fast inclusions are also demonstrable inside the cells of sweat glands, even in normal skin and these must also be differentiated

Unna (1896) regarded such granules as 'bacilli altered by the secretion of the coil glands' but their presence in normal skin calls for a modification of this view

Uncertainty still exists regarding the exact nature of the cellular response to invasion of the tissues by *M lepræ*. This uncertainty is largely due to the confusing terminology which has accumulated with reference to macrophage cells, particularly those of the connective tissues. We have referred to a proliferation of endothelial or endothelial-like cells including therein the true endothelium of the blood capillaries and lymphatic channels and also the morphologically similar cells lying



PLATE V



Fig 6—Cellular proliferation round a hair follicle Eyepiece No 7X Objective 1/6th inch



Fig 11—Fibrosis round hair follicle—scar type or depigmented patch. Eyepiece No 7X Objective, 1/6th inch



PLATE VI

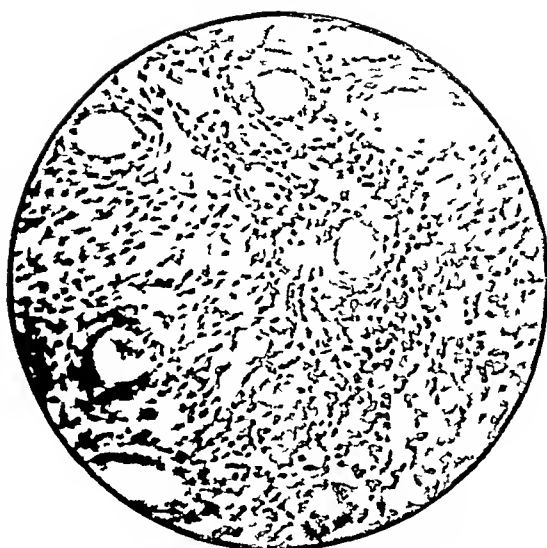


Fig 7—Cellular proliferation on round sweat gland  
Eyepiece No 2 Objective, 1/6th inch

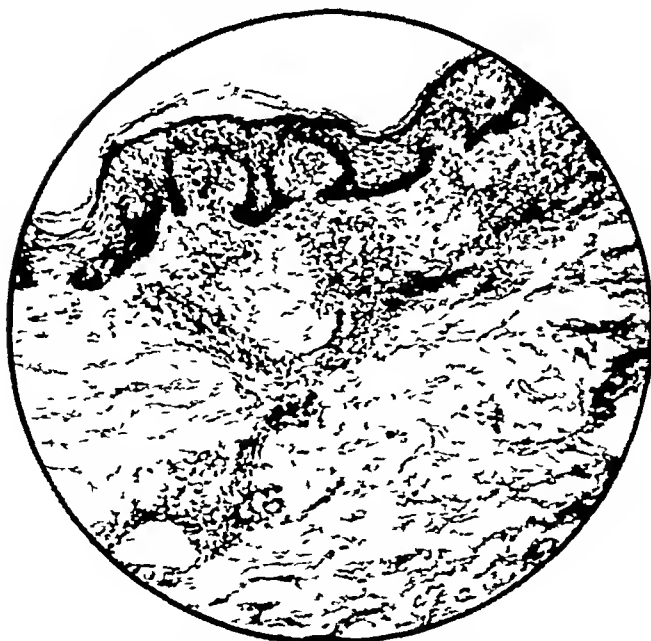


Fig 8—Cellular proliferation in papillae of corium with  
infiltration along lymphatic plexuses of corium Eyepiece  
No 15 Objective, 2/3rd inch.



free in the connective tissue. These last may be referred to as *wandering endothelial phagocytes* and Sabin in her latest work derives them definitely from the endothelial cell. These are the cells which in more advanced lesions of the nodular type become crammed with the specific organism of the disease, assume the well known 'foamy' appearance, and are then usually denominated 'lepra cells'.

#### (5) RELATIONSHIP BETWEEN CLINICAL PICTURE AND HISTO-PATHOLOGICAL APPEARANCES

When we come to the correlation of the pathological with the clinical findings, we are on much more difficult ground largely owing to lack of precise knowledge regarding the anatomy and physiology of the skin.

(a) *Depigmentation*—The precise mode of pigment formation in the skin is still under debate. Briefly there are two views, (i) that held by English and certain American dermatologists to the effect that the formation of the normal skin pigment, melanin, takes place in the basal layer of the epithelium, (ii) the view of Bruno Bloch (1917), of Acton (1922), and of certain of the continental observers that pigment formation is a function of certain specialized cells, the melanoblasts, lying in the upper part of the corium, the pigment reaching the epithelium by lymphatic drift. Recent work has demonstrated that melanin or its precursor is a derivative of protein decomposition and hence it is closely bound up with intestinal metabolism. Disturbances in pigment formation may therefore result from abnormalities in two directions, (i) abnormalities of protein metabolism, (ii) interference with the transport of the pre-melanin substance or substances to the melanoblasts—or to the cells of the basal layer of the epithelium. So far as we can judge, there is no interference with protein metabolism in the early stages of leprosy: the partial depigmentation of the lesions under discussion would appear to be due to the mechanical action of the proliferated endothelial cells interfering with the blood and lymph supply to particular areas and hence interfering also with normal pigment deposition in these areas.

(b) *Nerve disturbances*—The varieties of sensory disturbances most commonly met with are hyperæsthesia and paræsthesia—either or both of which may be present before any lesion is definitely present—followed by analgesia, anæsthesia to light touch, and loss of thermal sensation. Presumably there is irritation of the sensory receptors in the skin followed by inhibition of these structures as the disease spreads in the skin and up the fine nerve terminals. The early onset of analgesia is a feature of interest: it might be imagined that pain being a form of 'protopathic' sensibility would not be affected at such an early date as the sensations of light touch and slight temperature discrimination which are subserved by the more highly differentiated 'epicritic' fibres. There are two possible explanations. (1) The nature of the pain receptor which is believed to exist as a free axon termination without a surrounding capsule, thus differing from the fibres subserving other cutaneous sensations which commonly end in more complicated end-organs. (2) The second possible explanation is provided by the recent work of Adrian (1926). This worker has shown that the pain receptor is characterized

by a very brief duration of the discharge which results from weak stimuli he suggests that these brief discharges may not be adequate to evoke the pain response, but may serve instead for momentary sensations of contact. We may imagine, therefore, that the pain receptors, lacking the protection afforded to the other receptors by the possession of a surrounding capsule, are damaged at an earlier date by the leprotic cellular proliferation in the papillæ of the corium. The threshold of adequate stimulation is raised and stimuli which should be interpreted as painful are interpreted as sensations of contact only.

(c) *Hair follicle and gland disturbances*—Distortion of hair follicles and absence of sweat and sebaceous secretions are explicable on purely mechanical grounds, viz, the marked cellular proliferation around these structures leading to interference with their nutrition and function.

(d) *Hyperkeratosis and parakeratosis*—Irregularities in cornification are partly of 'central' and partly of 'peripheral' origin. Where the condition is a mild one the explanation is probably to be found locally, viz, as a result of deficient blood supply to the particular area. Where, however, there is an ichthyotic-like condition there is probably in addition depressed thyroid function. Depressed thyroid function can be detected clinically, particularly in the colder periods of the year, in an appreciable proportion of cases.

### SUMMARY

In this paper an attempt has been made to present a description of the depigmented patch in leprosy from the clinical and pathological viewpoints. The relationship of this particular type of lesion to the disease as a whole is discussed. The different clinical varieties of patch are then dealt with together with a brief reference to certain attributes of this type of lesion. The histological changes are next reviewed and lastly an attempt is made to correlate the clinical picture with the histological findings.

My thanks are due to Lieut-Col H W Acton, I.M.S., the Director, the School of Tropical Medicine and Hygiene, Calcutta, for helpful suggestions during the course of this investigation and to Dr E Muir, M.D., F.R.C.S. (Edin.), in charge of Leprosy Research for permission to publish this paper.

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# STUDIES ON THE SEDIMENTATION OF ERYTHROCYTES

BY

JOHN M HENDERSON, M B, Ch B (Glas),

*Working under the British Empire Leprosy Relief Association at the School of Tropical Medicine and Hygiene, Calcutta*

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THE phenomenon of the sedimentation of erythrocytes, noted and described by John Hunter and others over a hundred years ago and adapted to clinical uses by Fåhræus (1921) has found a wide application in the study of different physiological and pathological conditions. Perusal of the available literature does not reveal any extensive investigation into the effect of external temperature on this phenomenon. Gram (1921) states that 'the sedimentation increases with the surrounding temperature till 30 Celsius'. This statement is based on the examination of two pathologic double specimens kept respectively at 20 and 28 Celsius. It was decided to investigate this point more fully.

*Methods*—We employed a modification of the technique now used as a routine in the Leprosy Research Laboratory (Mun 1928). 0.6 c.c. of a 5 per cent solution of sodium citrate in distilled water is placed in a 5 c.c. all glass syringe and to it is added 2.4 c.c. of blood from the median basilic vein, 1 c.c. of the blood citrate mixture is taken in each of two 1 c.c. pipettes graduated in 1/100ths of a c.c. The pipettes are placed in suitable racks and their lower ends are plugged by being inserted into holes bored in rubber corks. One of the pipettes is kept at room temperature (which varied from 8°C to 22.5°C during the course of these investigations), while the other is placed in the incubator at temperatures varying from 35° to 37°C. The top level of the erythrocytes is read off every ½ hour up to 2½ hours (i.e., 5 readings in all) and the mean of readings 3 and 5 (at 1½ hours and 2½ hours respectively) multiplied by 10 is taken as the index of sedimentation.

In all, 90 samples of blood were tested by this duplicate method, of these 59 were obtained from cases of leprosy of different types and stages, while the remaining 31 were obtained from cases of kala-azar, tuberculosis (pulmonary and gland), syphilis, skin disease and dog-bite.



Table I shows the frequency distribution of the sedimentation indices of the 90 samples, the readings being taken at room temperature ( $8^{\circ}$ – $22.5^{\circ}\text{C}$ )

TABLE I

Frequency distribution of sedimentation indices of 90 samples of blood at room temperature ( $8^{\circ}$ — $22.5^{\circ}\text{C}$ )

Sedimentation index classes	Mid point of sedimentation class	Frequency (Z)	Deviation from origin in class units (X)	Zx	Zx <sup>2</sup>
0—9	4.5	13	0	0	0
10—19	14.5	10	1	10	10
20—29	24.5	6	2	12	24
30—39	34.5	11	3	33	99
40—49	44.5	14	4	56	224
50—59	54.5	17	5	85	425
60—69	64.5	11	6	66	396
70—79	74.5	8	7	56	392
TOTALS (S)		90		318	1,570

From this table we can derive the following statistics —

(1) Moments about the arbitrary origin at a sedimentation rate of +5 ( $V_1$  and  $V_2$ )

$$V_1 = \frac{S(Zx)}{S(Z)} = \frac{318}{90} = 3.533333$$

$$V_2 = \frac{S(Zx^2)}{S(Z)} = \frac{1570}{90} = 17.444444$$

(2) Moment about the mean  $\tau_2$

$$\tau_2 = V_2 - (V_1)^2 = 17\,444\,444 - 12\,482\,089$$

$$= 4\,962\,355$$

(3) Corrected moment about the mean ( $\mu_2$ ) = moment about the mean—  
Sheppard's correction for grouping

$$= \pi_2 - 0.083333$$

$$= 4.962355 - 0.083333 = 4.879022$$

(4) Mean

Sedimentation index at point of arbitrary origin	= 45
Number of class units from origin to mean ( $V_1$ )	= 3533
Number of sedimentation indices per class	= 10

$$\text{Mean sedimentation index for group} = 45 + \frac{35 \times 33}{35 + 33} = 39.83$$

(5) Standard deviation ( $\sigma$ ).

$$\sigma = \sqrt{\mu_2} = \sqrt{4879022} \text{ in class units} \\ = 2208858 \text{ in class units}$$

Number of sedimentation indices per class = 10

$$\therefore \sigma = 2208858 \times 10 = 22089$$

(6) Probable error of mean =  $\frac{\text{standard deviation}}{\sqrt{\text{number of observations}}}$

$$= \frac{22089}{\sqrt{90}} = 2328$$

(7) Mean sedimentation index for group =  $39.83 \pm 2.328$

Table II shows the frequency distribution of the sedimentation indices of duplicate samples from the same group kept in the incubator at temperatures from  $35^\circ\text{--}37^\circ\text{C}$

TABLE II

*Frequency distribution of sedimentation indices of 90 samples of blood at incubator temperature ( $35^\circ\text{--}37^\circ\text{C}$ )*

Sedimentation index classes	Mid point of sedimentation class	Frequency (Z)	Deviation from origin in class units (X)	Zx	Zx <sup>2</sup>
0—9	4.5	6	0	0	0
10—19	14.5	11	1	11	11
20—29 ..	24.5	9	2	18	36
30—39 .	34.5	18	3	54	162
40—49	44.5	12	4	48	192
50—59 .	54.5	9	5	45	225
60—69 ..	64.5	15	6	90	540
70—79 .	74.5	7	7	49	343
80—89	84.5	3	8	24	192
TOTALS (S)	.	90	..	339	1,701

From this table, by similar methods of calculation we derive the following statistics —

$$V_1 = 3766666$$

$$V_2 = 18900000$$

$$\pi_2 = 4709711$$

$$\mu_2 = 4626378$$

$$\text{Mean} = 42.17$$

$$\sigma = 21.509$$

$$\text{Probable error of mean} = 2.267$$

$$\text{Mean sedimentation index for group} = 42.17 \pm 2.267$$

Taking the means of the two series of samples, viz,  $39\ 83 \pm 2\ 328$  and  $42\ 17 \pm 2\ 267$ , the difference between these figures is not in a statistical sense, significant a study of the theory of probability teaches that the probable error of the difference between any two independent quantities is equal to the square root of the sum of the squares of the probable errors of the quantities entering into the difference

In this case, difference =  $42\ 17 - 39\ 83 = 2\ 34$ , square root of sum of squares of probable errors of these quantities

$$\sqrt{(2\ 267)^2 + (2\ 328)^2} = \sqrt{10\ 558873} \\ = 3\ 249$$

The difference between the two means  $42\ 17$  and  $39\ 83$  falls, therefore, within the limits of the probable error of these means and hence is not significant Putting the problem in another way—in the 90 samples of blood tested simultaneously in duplicate, a rise of temperature varying from a minimum of  $15\ 5^\circ\text{C}$  to a maximum of  $29^\circ\text{C}$  did not cause a significant acceleration in the sedimentation rate of the erythrocytes

Table III shows the 90 duplicate samples divided into 3 groups—'normal reaction,' 'equal reaction' and 'paradoxical reaction' By 'normal reaction' is meant that the sedimentation index of the incubator sample was higher than that of the sample of the same blood tested simultaneously at room temperature, 'equal reaction' means that the indices were equal and 'paradoxical reaction' that the index of the incubator sample was lower than that of the sample simultaneously tested at room temperature

TABLE III

(a) Normal reaction	58	$\begin{cases} 40 \text{ leper} \\ 18 \text{ non-leper} \end{cases}$
(b) Equal reaction	2	$\begin{cases} 1 \text{ leper} \\ 1 \text{ non-leper} \end{cases}$
(c) Paradoxical reaction	30	$\begin{cases} 18 \text{ leper} \\ 12 \text{ non-leper} \end{cases}$

#### Percentages

Normal reaction	$\begin{cases} \text{leper} \\ \text{non-leper} \end{cases}$	$\begin{cases} = 67.8 \text{ per cent} \\ = 58.1 \text{ } \end{cases}$
Abnormal reactions (b) + (c)	$\begin{cases} \text{leper} \\ \text{non-leper} \end{cases}$	$\begin{cases} = 32.2 \text{ } \\ = 41.9 \text{ } \end{cases}$

The high percentage of 'abnormal reactions' in the non-leper series is probably to be accounted for by the fact of this series being rather weighted by the inclusion of a large number of kala-azar cases (20 out of the total of 31) Kala-azar is a disease associated with profound changes in the blood plasma These 'abnormal

reactions' appear to be surprisingly constant in some cases, the most striking example of this occurred in a patient suffering from moderately advanced leprosy (B2 by Mun's classification). Seven sets of duplicate samples of blood were examined over a period of 6 weeks and in every instance the sedimentation index was higher at room than at incubator temperature, i.e., the red cells sedimented more markedly at room than at incubator temperature.

Taking the sedimentation index in any particular case as the mean of the  $1\frac{1}{2}$  and  $2\frac{1}{2}$  hour readings at room temperature multiplied by 10, Table IV shows the frequency distribution of the 32 'abnormal reactions' by sedimentation index classes.

TABLE IV

*Frequency distribution of 32 'abnormal reactions' by sedimentation index classes*

Sedimentation index classes	Frequency
0—9	2
10—19	0
20—29	1
30—39	4
40—49	7
50—59	10
60—69	5
70—79	3
TOTAL	32

We next attempted to find out why 'abnormal reactions' should occur so relatively infrequently (3 times out of 32 instances) in bloods with sedimentation indices 0—29 by our notation. For this purpose a fresh batch of 32 cases (24 lepers and 8 dog-bite cases) was selected and simultaneous estimations made of the sedimentation rate of the erythrocytes and of the viscosity of the blood serum—both at room temperature. In this connection it should be noted that the temperature co-efficient of viscosity is deliberately omitted, to have applied a correction for temperature to the viscosity readings would naturally have invalidated the sedimentation readings taken at corresponding temperatures. Using a modified Ostwald viscometer, it was found that with the particular instrument in use the time of outflow varied from a minimum of 24.0 to a maximum of 32.9 seconds. The group was then divided into 2 approximately equal parts, viz., (a) comprising those cases in which the viscosity readings fell between 24 and 28.9 seconds, and (b) those in which the readings fell between 29 and 32.9 seconds.

In group (a) there were 15 cases and in this group the simultaneously recorded sedimentation readings showed a mean figure of  $10.50 \pm 2.145$ . In group (b) comprising the remaining 17 cases, the mean of the simultaneously recorded sedimentation readings was  $54.30 \pm 5.657$ . Now it is well known that the viscosity of colloids and hence of proteins, is at a minimum at the iso-electric point, hence from the figures quoted above, sedimentation indices in the neighbourhood of  $10.50 \pm 2.145$  by our notation occur in samples of blood the sera of which are iso-electric or nearly so.

Turning again to Table IV it will be noticed that the tendency towards the occurrence of 'abnormal reactions' varies inversely as the distance from the iso-electric point. It will be noticed from the table, however, that in the upper ranges of sedimentation indices (from about 70 upwards) there tends to be a diminution in the number of 'abnormal reactions'. The maximum sedimentation index that we have obtained in this laboratory over a large series of investigations is 81 in our notation. In cases, therefore, in which the sedimentation index at room temperature is 70 or over, the possible effect which an increase in temperature might have is masked by a purely mechanical factor, viz, the very marked 'packing' of the red cells which occurs at these high ranges.

We next determined to find out the particular point at which the maximum acceleration of red cells occurred. Tables V and VI show the frequency distribution of the maximum acceleration in the 90 samples investigated at room temperature and at incubator temperature respectively. The temperature did not vary more than  $1^{\circ}\text{C}$  during the duplicate test of any particular sample. It will be noticed from the tables that the figures shown therein exceed the total number of samples tested. This is due to the fact that in 7 instances at room temperature and in 11 instances at incubator temperature there was an equal 'maximum rise' during two different (and usually consecutive) half hour periods. It is obvious from the tables that in samples with a low sedimentation index the maximum sedimentation rate occurs towards the end of the observation period, while the converse holds true with regard to samples with a high sedimentation index. In other words, the distance of the point of maximum sedimentation from the time of commencement of the experiment varies inversely as the sedimentation index of the particular sample. This holds true whether the readings are taken at room temperature or at the temperature of the incubator. The phenomena are represented graphically in the figure below which represents the consecutive half hour readings at room temperature of two samples of blood, one with a high sedimentation index (a), the other with a low index (b). These are typical of the appearances presented in other cases.

#### SUMMARY AND CONCLUSIONS

(1) The statement that rise of temperature causes an acceleration in the sedimentation rate of erythrocytes does not in every instance hold true for samples of blood obtained from pathological cases. Ninety samples of blood from patients suffering from naturally acquired disease or from the effects of injuries were

tested in duplicate, one sample being put up at ordinary room temperature, the other in the incubator. In 32 instances (approximately 38 per cent), the rate of sedimentation of the erythrocytes at incubator temperature was either equal to (2 cases) or less than (30 cases) that of the same sample of blood simultaneously tested at room temperature. The tendency for this abnormal type of reaction to occur seems to be roughly in inverse ratio to the deviation of the particular sample from its iso-electric point. In the absence of further evidence, it is idle to speculate on the intimate cause of the electric change which occurs in those cases showing an abnormal type of reaction. It seems possible that it is due to some alteration in the relative or absolute distribution of the plasma proteins. Work is in progress along these lines and it is hoped to report thereon at a future date.

(2) The distance of the point of maximum sedimentation rate from the time of commencement of observation varies inversely as the sedimentation index of the sample.

(3) Where it is desired to trace the variations in the sedimentation index of the blood of any particular case over a period of time, it is desirable that the successive observation should be made at constant temperatures.

My thanks are due to Dr E. Muir who is in charge of the Leprosy Research Laboratory for permission to publish this paper, to Mr N. K. De, B.Sc., the chemist to the department for valuable assistance during the course of these investigations, and to Dr L. Everard Napier and Dr E. C. R. Fox for permission to investigate cases under their care in the Carmichael Hospital for Tropical Diseases and in the Pasteur Institute respectively.

TABLE V

*Frequency distribution of maximum acceleration of red cells, 90 samples, at room temperature (8°—22.5°C)*

Time

	1st half hour	2nd half hour	3rd half hour	4th half hour	5th half hour
0—9	.		1	4	10
10—19		2	2	6	2
20—29		1	4	1	
30—39	1	9	3		
40—49	9	6			
50—59	12	5			
60—69	8	3			
70—79	7	1			

Total = 97. Seven cases showed 'equal maximum' at 2 different half hours.

TABLE VI

Frequency distribution of maximum acceleration of red cells, 90 samples, at incubator temperature ( $35^{\circ}$ — $37^{\circ}\text{C}$ )

Time

Sedimentation Index		1st half hour	2nd half hour	3rd half hour	4th half hour	5th half hour
	0—9			2	2	5
	10—19		2	4	6	3
	20—29		3	5	1	
	30—39		11	7	1	
	40—49	3	8	2		
	50—59	3	7			
	60—69	5	11			
	70—79	6	1			
	80—89	3				

Total = 101 Eleven cases showed 'equal maximum' at 2 different half hours

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# A CHOLERA AND DYSENTERY BACTERIOPHAGE

BY

LIEUT-COLONEL J MORISON, I M S ,

AND

MILITARY ASSISTANT SURGEON A C VARDON, C P H (Harvard),  
*King Edward VII Memorial Pasteur Institute and Medical Research Institute,  
Shillong, Assam*

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DURING 1927 at Rangoon Major C de C Martin, I M S , and one of the writers (J M ) isolated from a case of cholera a strain of bacteriophage active against the patient's strain of *V cholerae*, a laboratory strain of *V cholerae* and a laboratory strain of *B dysenteriae* (Shiga) This suggested the cultivation of a combined bacteriophage for cholera and dysentery Such a bacteriophage seemed desirable, for the best time to administer bacteriophage is early in the illness as the initial diarrhoea of acute dysentery and the first symptoms of cholera are not very unlike

The medium used for cultivating the bacteriophage is a bouillon made by digesting goat's flesh freed from fat with dried papain (obtainable from Messrs Tarrant & Co , Colombo) It is for this purpose superior to any other medium we have tried A description of its preparation was given by Major Martin(1), but it may be convenient if we give the slightly modified procedure now in use at this laboratory

Seventy-five grammes of dried papain and 600 grammes of minced goat's flesh free from fat are ground together and water is slowly added to make up the whole to 3,600 millilitres This is placed in a large flask and three or four such flasks are put in a water bath The water bath may be heated with an electric heater having a thermostatic control, but we find a copper coil through which steam is passed more efficient The temperature of the flasks is raised 10°C every hour till 80°C is reached After a further hour at 80°C the temperature is raised to boiling point and sufficient N/1 NaOH is added to bring the reaction to pH 7.8 The digest is allowed to cool and is then filtered through a thick cloth and through filter paper, and is autoclaved for 45 minutes



at 15 lbs pressure For use this stock bouillon is diluted with two parts of water The final product must be perfectly clear We no longer add the meat infusion described by Major Martin, for we found that, when the meat infusion was added, the growth of the *V cholerae* was apt to alter the pH of the medium so much that the action of the bacteriophage was affected

The strains of *V cholerae* used in this Institute for growing and testing bacteriophage are at present twelve in number, and for convenience are known as strains A, B, C, D, E, F, G, H, J, K, L, M

TABLE I

Strains	Source
A	From Central Research Institute, Kasauli
B	} From School of Tropical Medicine, Calcutta
C	
D	
E	} From Major R H Malone, RMS strains isolated when he was working on cholera with Dr D'Herelle
F	
G	
H	From Central Research Institute, Kasauli, a strain used at that Institute for making high titre serum
J	} Isolated during a recent epidemic in Shillong
K	
L	
M	

A dysentery bacteriophage in which had been incorporated the original cholera dysentery bacteriophage referred to above, was brought by one of us (J M) from Rangoon This strain of bacteriophage completely lysed strain H, the remaining strains were not visibly affected This bacteriophage was, however, very active against our laboratory strains of dysentery (Shiga, Flexner and Y of Hiss and Russell) Three strains of cholera bacteriophage moderately active respectively on E, F and G vibrios were kindly supplied by Major R H Malone, RMS None of these had any action on any of our strains of *B dysenteriae*

After eight successive sowings and filterings on their respective vibrios, bacteriophage strains E, F and G became very active

Three broth suspensions of vibrios E, F and G lysed by their homologous bacteriophage were now mixed and filtered This being the ninth passage was called bacteriophage No 9 and was found to be active not only against vibrios E, F and G, but also against strain A The activity of the mixed filtrate was

Bacteriophage No 9 versus vibrio	A	complete lysis	+++
"	"	"	E slight lysis +
"	"	"	F moderate lysis ++
"	"	"	G complete lysis +++

Two further passages on the above vibrios were made, and on each occasion the lysed cultures were mixed before filtration To the eleventh filtrate the cholera

dysentery bacteriophage from Raugoon was added and this formed bacteriophage No 12. The further steps in the building up of the combined bacteriophage are shown in Table II. It will be noted that No 12 completely lysed vibrio strains A and G had a moderate action on *B. dysenteriae* 'Y' and vibrio F and a slight action on Flexner and vibrio E. The filtrate of all six completely lysed Flexner and vibrios A and G. The lysis of vibrio E was somewhat better at the 16th passage when *B. dysenteriae* Shiga was introduced in the passages and all three of the *B. dysenteriae* and three of the vibrio strains were completely lysed. At the 18th passage, vibrio E, against which the bacteriophage had been acting irregularly, was omitted.

At the 19th passage vibrios F and G were omitted and the procedure was continued with the susceptible strains A, H and J. When the 31st passage had been accomplished, the filtrate was tested against the whole series except K which at the time was found to be contaminated.

Bacteriophage No 15 was used as seed for the production of a combined dysentery cholera bacteriophage. This was employed in an epidemic of cholera that broke out in Shillong. It will be noted that seed 15 happened completely to lyse vibrio J, the first vibrio isolated during the epidemic.

The epidemic consisted of 27 cases, of these 21 were not given bacteriophage and 16 died. Six unselected cases were given bacteriophage and one died. This case when he received bacteriophage was *in extremis*.

Three cases are worthy of note. The first case in which we had an opportunity to test the bacteriophage was (case 4 of the series) admitted to the Military Hospital under Captain Rosenbloom, I M S, with severe vomiting, large, frequent rice-water stools, cramps and collapse. He was given intravenous hypertonic saline to which 2 c.c. of bacteriophage was added and he received 2 c.c. of bacteriophage in water by the mouth every four hours. Within 18 hours he had recovered and appeared so well that a diagnosis of cholera was doubted. Cholera vibrios, strain J, were recovered from his stool on the first day of his illness.

L, a boy aged 7 years whose sister had died of cholera 3 days before, took violently ill at 2 P.M. on the 7th July. At 4 P.M. he was seen at his home by one of us (J.M.) vomiting incessantly and passing copious rice-water stools. He received 2 c.c. of bacteriophage by the mouth at 4-30 P.M., at 6 P.M. he was admitted to hospital and got a second dose. The medical officer of the hospital saw him at 7-30 P.M., but the vomiting and purging had ceased, the pulse was good and the usual transfusion was postponed. The patient had a good night, there were no stools the following day and he made an uninterrupted recovery. Vibrio K was isolated from this patient.

J.B.T. was a friend of a patient in the cholera ward and was attending to him. About 2-30 P.M. on the 11th July he was seized with colic, vomiting and passed two large copious stools. The compounder on his own initiative administered 2 c.c. of bacteriophage and a second dose two hours later. The vomiting ceased within an hour of the first dose, and by the time the medical

TABLE II

Passage number of Bacteriophage	Dysentery Shiga	Dysentery Flexner	Dysentery Y	Cholera A	Cholera B	Cholera C	Cholera D	Cholera E	Cholera F	Cholera G	Cholera H	Cholera J	Cholera L	Cholera M
31	+	+	+	+	+	+	+	+	+	+	+	+	+	+
28	+	+	+	+	+	+	+	+	+	+	+	+	+	+
27	+	+	+	+	+	+	+	+	+	+	+	+	+	+
26	+	+	+	+	+	+	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	+	+	+	+	+	+	+
23	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+
19	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Note — (1)

0 Test not carried out

— No lysis

+ Slight lysis

++ Moderate lysis

+++ Broth culture quite clear and remaining clear till filtered the next day

++++ Broth culture quite clear and remaining clear for four days.

officer paid his visit in the evening the colic and purging had ceased and the patient was apparently well. Cholera vibrios were present in the stool on the first evening.

Cholera broke out in a small village in Goalpara on the 5th November, 1928. Between that date and the 27th November there were 33 cases. On the 20th November one of our staff Dr Pal Choudhury arrived at the village and found that six persons had died and two were convalescent. Thirteen cases had been ill for 2 to 7 days, three were moribund, five others were almost pulseless. These thirteen sick were given bacteriophage. The moribund cases and two others died. After Dr Pal Choudhury's arrival there were 12 more cases, of these 3 died. The epidemic may be summarized —

	Cases	Deaths
Cases that received no bacteriophage	8	6
Cases ill before bacteriophage was available but given bacteriophage on 20th and following days	13	5
Cases given bacteriophage on the 1st day of illness	12	3

No other treatment was available during this epidemic and the patients remained in their own huts.

The two epidemics give the following results —

	Cases	Deaths	Mortality per cent
Cases having no bacteriophage	29	22	75.8
Cases receiving bacteriophage (including 4 moribund)	31	9	29.0

In May 1928 before we had an opportunity of testing the combined bacteriophage on cases of cholera, Dr Pal Choudhury visited a remote Khasi village severely stricken with dysentery.

The details of this epidemic were reported to Government and formed the subject of a paper read by one of us at the recent annual meeting of the Assam Branch of the British Medical Association (2).

In dealing with this epidemic Dr Pal Choudhury employed at first our dysentery bacteriophage and subsequently the combined cholera dysentery bacteriophage. The results, under the adverse conditions existing in this jungle village, where no milk was available, were very satisfactory. Dr Choudhury returned with the impression that the combined bacteriophage was even more effective than the dysentery bacteriophage.

In individual cases of dysentery in Shillong we, too, have come to the opinion that the combined bacteriophage is as reliable as the dysentery bacteriophage which we ordinarily use.

In our hands a seed does not maintain its activity indefinitely. As soon as a falling off is noticed, the use of this seed is discontinued and it is subcultured until its activity is regained. Thus in the series in Table II only seed strains Nos 15, 17, 21, 22 and 31 have been used for the preparation of therapeutic bacteriophage.

Therapeutic bacteriophage for cholera and dysentery is prepared by making suspensions of *B. dysenteriae* Shiga, Flexner, Y of Hiss and Russell, Sonne and four strains of *V. cholerae*, all sown in different flasks

Each flask contains 900 c.c. of papain broth to which is added the whole washings of an 18 hours' agar culture of the micro-organism concerned. To each of the seven flasks, three loopfuls of the seed bacteriophage are added, the flasks are thoroughly shaken and incubated at 37°C for six hours. After six hours lysis must be complete in all seven flasks. The flasks are then removed from the incubator, again shaken and left at room temperature overnight. The contents of the flasks are then emptied into large four-litre flasks, thoroughly mixed and transferred to tall cylinders from whence they are filtered through Pasteur Chamberland candles 'F' under a vacuum not exceeding 6 lbs.

When filtration is complete the tubing on either side of the filter flask is clamped and removed from the filter candle on the one side and the pump on the other, the ends of the rubber tubing are flamed and are surrounded with sterile cotton-wool. The flasks are now incubated for four days. The contents of each flask, if still perfectly clear, are then bottled in ampoules. The ampoules in turn are incubated for 48 hours at 37°C and are examined for clarity. The titre of the bacteriophage is now tested against *B. dysenteriae* Shiga and one of the strains of *V. cholerae*. A combined bacteriophage must produce complete lysis in a dilution 1 in 10<sup>7</sup>. The method is that described by D'Herelle(3). The ampoules are stored in a refrigerator till issued.

Immediately after use the candles are boiled in water, dried in the incubator and heated to a dull red heat in a muffle furnace. After cooling, the candles are tested for leakage against an air pressure of 10 lbs. Any candle showing the slightest air leak is discarded. The candles are then connected by pressure tubing to one litre conical filter flasks. The flask and the candle are then sterilized together in the autoclave.

We do not discuss here the question whether the combined bacteriophage is a mixture of different strains or whether it is one strain which has acquired polyvalent activities.

Eight thousand nine hundred and fifty-nine doses of dysentery bacteriophage and twenty-four thousand six hundred doses of combined bacteriophage have been issued since the 15th of April and reports from reliable sources are beginning to come in.

### SUMMARY

- 1 The procedure is described which led to the production of a seed bacteriophage active against eleven strains of *V. cholerae* and types of *B. dysenteriae*.
- 2 This bacteriophage has therapeutic value in both cholera and dysentery.
- 3 The technique of the manufacture of the combined cholera-dysentery bacteriophage of therapeutic use is described.

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# AN EXPERIMENTAL STUDY IN HÆMOLYSIS

BY

KSHITISH CHANDRA SEN, D SC ,

*Department of Chemistry, Allahabad University,*

AMARESH CHANDRA ROY, M SC ,

*Research Scholar, University of Allahabad,*

AND

NARENDRA NATH MITRA, M SC ,

*Chemist, East Indian Railway Laboratory, Allahabad*

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## INTRODUCTION

A LARGE number of workers have studied the hæmolysis of red blood corpuscles by different substances [Hobei (1924)] but as yet no clear explanation exists as to the mechanism of the change in the permeability of the corpuscle membrane under the influence of different hemolytes. That a single explanation may not cover all cases is evident from the fact that pure water alone will hæmolyse the corpuscles which is entirely due to the absorption of water owing to a difference in the osmotic pressure inside and outside the cell membrane. The membrane is thus ruptured owing to a swelling of the whole cell.

According to modern conceptions, the membrane of red blood corpuscles is a skeleton containing protein together with some lipid materials. Hattori (1921), Gough (1924) and others assumed that the membrane consists mainly of lecithin and cholesterol. Pascucci considers that it is a proteid permeable membrane with a large amount of lecithin, cholesterol and cerebroside. Since it is well known that all the constituents of the corpuscle membrane are colloidal in nature, it is evident that the permeability of the membrane will be largely influenced by the action of substances which may affect the colloidal properties of any of the constituents. In the present paper a mechanism of hæmolysis will be suggested on the basis of the colloidal properties of the membrane. The present paper also contains an experimental study of hæmolysis by various chemical hemolysers as well as by hæmolytic serum. A study has also been made of the effect of mixtures

of hæmolytes and that of the action of normal serum in presence of other hæmolyzers. The experiments have been done both in saline as well as in sucrose solution.

### EXPERIMENTAL METHOD

The method of experimentation depends upon the colorimetric determination of complete hæmolysis. When the end-point was observed visually and when care was taken to select glass tubes of the same bore and thickness and of equal transparency, practically concordant results could be obtained in duplicate experiments, the error being very slight. In all the experiments given in these pages, defibrinated sheep corpuscle, washed three times with either normal saline or sucrose as the case may be to free it completely from serum, has been used. The solution of chemical hæmolytes such as sodium taurocholate, potassium oleate and saponin were all prepared fresh, the substances being pure chemicals of either Merck or Kahlbaum. Since the experiments have been carried on through a long period, it has been found necessary to use fresh samples of blood many times, consequently in different tables identical data on hæmolysis will not be observed. The results of each table, however, are absolutely comparative and no difficulty will be experienced in the proper interpretation of the data. The usual method of carrying out an experiment has been to take a certain amount of the corpuscles in one test-tube, and in another test-tube a known amount of hæmolyte mixed with calculated quantity of normal saline or sucrose as the case may be to give a constant volume when the corpuscles and hæmolyte solution were mixed together. The mixing was rapidly done three times, and then the mixture was allowed to rest in an uniform temperature of  $32^{\circ}\text{C}$  and the time of complete hæmolysis was then noted. The results obtained have been summarized and are given in different sections.

#### *I The nature of the time-dilution curves in taurocholate hæmolysis*

In an early paper on the hæmolytic action of bile derivatives, MacLean and Hutchinson (1909) made some interesting observations on the hæmolytic behaviour of the sodium salts of cholalic, choleic and glycocholic acids. They found that these substances are capable of producing hæmolysis in the ordinary way when strong doses are used but exhibit marked peculiarities when present in considerably weaker amounts. It was found that under similar conditions the same hæmolytic effect can be produced in a given time by widely divergent amount of the salt. Between these two points lies what may be termed a more or less neutral zone in which the hæmolysis is very considerably delayed depending on the relative amount of the hæmolytic agent employed. Ponder (1922) in recent years has shown, using low concentration of taurocholate and cells that the time-dilution curves of taurocholate is of much simpler nature and does not show any abnormal behaviour like that of glycocholate. We have made a thorough study of sodium taurocholate as a hæmolyte with different concentrations both of red blood corpuscles and of the hæmolyte. Our results show that sodium taurocholate also



shows a similar behaviour to that of glycocholate at higher concentrations, but at lower concentrations the time-dilution curve is perfectly normal. The results given in Table I have been obtained with 1 c.c. of a 5 per cent red blood corpuscles, the taurocholate concentration being 3 per cent and the total volume 5 c.c.

TABLE I

Taurocholate in c.c.	Final concentration	Time in minutes for complete hemolysis
0.1	1/1,666	26
0.5	1/333	3
1.0	1/166	16
1.5	1/111	11
2.0	1/83.3	27½
2.5	1/66.6	42
3.0	1/55.6	27
3.5	1/47.6	5

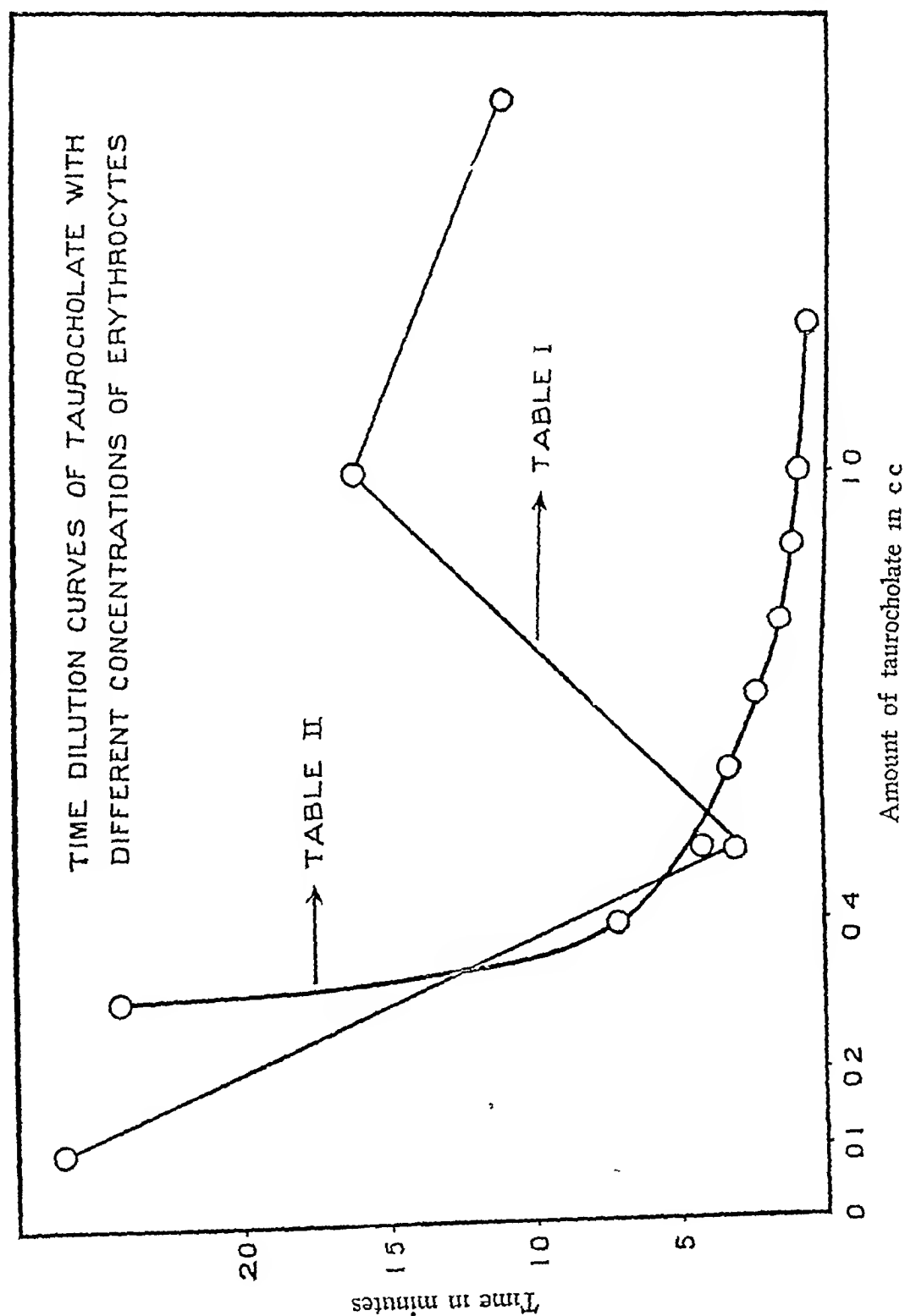
In Table II are shown the results with lower concentrations of corpuscles as well as of hæmolytes, the taurocholate concentration is 0.3 per cent and 1 c.c. of a 1 per cent emulsion of corpuscles has been used.

TABLE II

Taurocholate in c.c.	Final concentration	Time for complete hæmolysis
0.1	1/16,666	< 2 hours
0.2	1/8,333	< 2 hours
0.3	1/5,555	24 minutes
0.4	1/4,166	5 minutes
0.5	1/3,333	4 minutes
0.6	1/2,777	3 minutes 15 seconds
0.7	1/2,381	2 minutes 20 seconds
0.8	1/2,083	1 minute 30 seconds
0.9	1/1,851	1 minute 7 seconds
1.0	1/1,666	0 minute 55 seconds
1.2	1/1,389	0 minute 40 seconds

In Fig 1, the results given in Tables I and II are plotted. Though the scales are different, the nature of the curves will be immediately apparent from the graphical representation. It will be observed that with higher concentrations of

Fig. 1.



red blood corpuscle and taurocholate the time-dilution curve shows a sort of periodicity, while in low concentrations the series is perfectly normal. We have discussed these results in a separate paper elsewhere but the interesting thing which should be noticed here is that taurocholate hæmolysis is exactly similar in nature to that of glycocholate, and cholate hæmolysis when comparatively higher concentrations of corpuscles and hæmolyte are used. This also possibly explains some contradictory results to be found in the literature. Thus Ponder (1922) found that glycocholate is a very weak hæmolyte when compared to taurocholate, but we have found previously (Sen and Basu, 1928) that glycocholate is a much stronger hæmolyte than taurocholate. Thus it was observed by us that 1 c.c. of M/20 sodium glycocholate required one minute only for complete hæmolysis of a certain quantity of corpuscles, whereas under similar conditions 1 c.c. of M/20 sodium taurocholate required 42 minutes to effect this change. The difference between our results and that of Ponder probably depends upon the fact that we have been comparing the two hæmolytes in concentrations far different from that used by Ponder. It is quite possible that in the concentrations we have been using, the comparison has been made in that portion of the curves where the action of taurocholate was about the minimum but the effect of glycocholate was near about the maximum. This would show that glycocholate is a stronger hæmolytic agent. In the experiments of Ponder, however, the concentrations of the compared hæmolytes may have been such that the conditions are just the reverse of ours, hence an entirely different result was obtained. It, therefore, appears that sodium glycocholate may have either a greater or lower hæmolytic action than sodium taurocholate depending on the particular concentration range in which the comparison is made.

## *II Effect of mixtures of hæmolytes*

Though the study of hæmolysis by single chemical hæmolysers have been frequently made by different investigators, much investigation has not been carried out on hæmolysis by mixtures of chemical hæmolytes. In an early paper, Moore, Wilson and Hutchinson (1909) following the experiments of Sachs and Altmann (1908), studied what they called the balancing action of certain pairs of hæmolysers in preventing hæmolysis and showed that there was an inhibiting action observed when sodium linoleate was used in conjunction with a pig serum both of which have a hæmolytic action on sheep's corpuscles. They further observed that when a mixture of sodium oleate and linoleate was used, no such inhibition was to be observed. A little earlier to this paper, Arrhenius (1908) found that sodium oleate increases the action of cobra poison but diminishes that of saponin. We have made a detailed study of the hæmolytic behaviour of mixtures of several chemical hæmolytes and some results as well as the conclusions arrived at will be given here. In Table III, some results with a mixture of saponin and oleate are shown. The corpuscle concentration has been one c.c. of a 1 per cent suspension and the total volume 5 c.c., the concentration of saponin being 1/1000 and that of oleate being 1/10,000.

TABLE III

Saponin in c c	Time in minutes for hemolysis in presence of oleate, c c				
	0	0·1	0·2	0·3	0·4
0·0	—	No hemolysis in 1½ hours	No hemolysis in 1½ hours	No hemolysis in 1½ hours	80
0·2	48	21	10 mins 35 secs	3 mins 9 secs	1 min 10 secs
0·3	19 mins 9 secs	8 mins 5 secs	2 mins 25 secs	2 mins 0 sec	0 min 40 secs
0·4	11 mins 10 secs	4 mins 40 secs	1 min 34 secs	1 min 0 sec	0 min 35 secs
0·5	6 mins 35 secs	2 mins 4 secs	1 min 0 sec	0 min 46 secs	—
0·6	4 mins 2 secs	1 min 32 secs	0 min 50 secs	0 min 27 secs	—

The results given in the above table show that oleate has a definite effect on the hæmolytic action of saponin. Thus it will be observed from all the results given above that the addition of oleate increases greatly the hæmolytic action of saponin. This is contrary to the results reported to have been obtained by Arrhenius. Exactly similar results have been obtained with the mixtures of taurocholate and oleate, as well as of saponin and taurocholate. In every case, the action of mixture of hæmolyseis appear to be of an additive nature and in these particular cases there is no retarding action.

### III Effect of acid and alkali

While studying the hæmolysis by mixtures of hæmolytes we have also made a detailed study of the hæmolytic action of some hæmolyseis in acid or alkaline solution and some interesting results have been obtained. It has been found that in certain cases acids accelerate whilst alkali retards the hæmolytic action of certain hæmolytes, whilst in some other cases a reverse effect is observed. Thus two series of results in presence of hydrochloric acid and caustic soda may be given here. The concentrations of corpuscles and hæmolytes are the same as in the results given in Table III.

TABLE IV

Saponin in c c	Time of hæmolysis in presence of HCl, final concentration		
	0	N/10,000	N/5,000
0·0	—	No hæmolysis in 2 hrs	No hæmolysis in 2 hrs
0·2	21 mins 16 secs	18 mins 22 secs	4 mins 20 secs
0·3	10 mins 38 secs	5 mins 53 secs	1 min 26 secs
0·4	6 mins 27 secs	2 mins 43 secs	0 min 49 secs
0·5	2 mins 57 secs	1 min 17 secs	0 min 30 secs
0·6	2 mins 6 secs	1 min 0 sec	0 min 20 secs

TABLE V

Oleate in c.c.	Time of hæmolysis in presence of HCl, final concentration	
	0	N/5,000
0.0	—	No hæmolysis in 2 hrs
0.4	34 mins 4 secs	No hæmolysis in 1 hr
0.5	13 mins 50 secs	15 mins 23 secs
0.6	8 mins 1 sec	12 mins. 21 secs
0.8	2 mins 45 secs	10 mins 31 secs
1.0	1 min 40 secs	9 mins 42 secs

In Tables VI and VII, the hæmolytic action of the same hæmolytes in alkaline solutions are shown. In these results the concentrations of corpuscles were 1 c.c. of a 5 per cent and 1 per cent suspensions respectively, the total volume 5 c.c., saponin concentrations 1 in 1,000 and oleate 1 in 10,000

TABLE VI

Saponin in c.c.	Time of hæmolysis in presence of NaOH, final concentration	
	0	N/500
0.0	—	No hæmolysis in 2 hrs
0.5	50	156
0.8	12	73
1.0	10	56
1.2	4	50
1.5	3	21

blood cells. But if the serum is added to the cells before the addition of taurocholate, a great inhibition of a haemolysis is observed. In a study of this acceleration or retardation of haemolysis by normal serum, it was shown by us (Sen and Sen, 1928) that Ponder's conclusions could be possible only under special conditions, and experiments carried by us with comparatively higher concentrations of red blood cells all showed that normal serum in every case inhibited the haemolysis whether it was added to the red cells before or after the addition of the taurocholate. Ponder has however suggested (private communication), that this acceleration is to be observed only under some particular concentration ranges of the reacting substances. Since no thorough investigation on this particular problem has been published as yet, we have made a detailed study of this retardation and acceleration of haemolysis by normal serum and we have been able to confirm Ponder's suggestions in this line. Since this acceleration and retardation of a haemolysis by normal serum must be of great theoretical importance we shall give below some of our results in details, in the following experiments, the concentration of cells is one per cent, taurocholate 0.3 per cent, total volume 5 cc and concentration of serum is 1/10, the quantity of corpuscles have been varied in different experiments. All the experiments have been done in normal saline.

TABLE VIII

2 cc corpuscles are used in these experiments	Time for complete haemolysis
(1) 2 cc taurocholate + 1 cc saline added half a minute after the addition of taurocholate	3 mins 2 secs
(2) 2 cc taurocholate + 1 cc serum added half a minute after the addition of taurocholate	50 per cent haemolysis in 2 hrs
(3) 15 cc taurocholate + 15 cc saline added half a minute after the addition of taurocholate	17 mins 8 secs
(4) 15 cc taurocholate + 15 cc serum added half a minute after the addition of taurocholate	No haemolysis in 1 hr

TABLE IX

	Time for complete haemolysis
(1) 1 cc cells + 0.5 cc taurocholate	7 mins 20 secs
(2) 1 cc cells + 0.1 cc serum added before + 0.5 cc taurocholate	122 mins
(3) 1 cc cells + 0.2 cc serum added before + 0.5 cc taurocholate	60 per cent haemolysis in 3½ hrs
(4) 1 cc cells + 0.5 cc serum added before + 0.5 cc taurocholate	No haemolysis in 4 hrs
(5) 1 cc cells + 0.5 cc taurocholate + 0.1 cc serum added half a minute after the addition of taurocholate	57 mins 46 secs
(6) 1 cc cells + 0.5 cc taurocholate + 0.2 cc serum added half a minute after the addition of taurocholate	63 mins
(7) a wide zone of 15 cc taurocholate + 0.5 cc serum added after the addition of taurocholate where we have shown	1 min 42 secs

TABLE X

	Time for complete haemolysis
(1) 0.5 cc cells + 0.5 cc taurocholate	3 mins 21 secs
(2) 0.5 cc cells + 0.1 cc serum added before + 0.5 cc taurocholate	16 mins 16 secs
(3) 0.5 cc cells + 0.2 cc serum added before + 0.5 cc taurocholate	No haemolysis in $\frac{1}{2}$ hr
(4) 0.5 cc cells + 0.5 cc taurocholate + 0.1 cc serum added half a minute after the addition of taurocholate	14 mins 46 secs
(5) 0.5 cc cells + 0.5 cc taurocholate + 0.2 cc serum added half a minute after the addition of taurocholate	22 mins 28 secs
(6) 0.5 cc cells + 0.5 cc taurocholate + 0.5 cc serum added half a minute after the addition of taurocholate	1 min 4 secs
(7) 0.5 cc cells + 0.5 cc taurocholate + 1 cc serum added half a minute after the addition of taurocholate	0 min 32 secs

In the following tables the effect of serum on oleate haemolysis is shown, concentration of oleate is 1 in 10,000, the other conditions being the same

TABLE XI

	Time for complete haemolysis
(1) 1 cc cell + 1 cc oleate + 1 cc saline added half a minute after the addition of oleate	2 mins 52 secs
(2) 1 cc cell + 1 cc oleate + 0.1 cc serum added before the addition of oleate	10 mins 56 secs
(3) 1 cc cell + 1 cc oleate + 0.1 cc serum added half a minute after addition of oleate	10 mins 48 secs
(4) 1 cc cell + 1 cc oleate + 0.5 cc serum added before	No haemolysis in 2 hrs
(5) 1 cc cell + 1 cc oleate + 0.5 cc serum added half a minute after the addition of the oleate	70 per cent haemolysis in $2\frac{1}{2}$ hrs
(6) 1 cc cell + 1 cc oleate + 1 cc serum added half a minute after the addition of oleate	90 per cent haemolysis in 2 hrs

TABLE XII

Amount of cells used each time = 0.2 cc

Amount of oleate used each time = 0.3 cc

Time of complete haemolysis when one cc saline was added to a mixture of corpuscles and oleate one minute after the addition of the oleate

6 mins 9 secs

No.	Amount in cc of serum added one minute after the addition of oleate	Time of complete haemolysis
1	0.1	146 mins
2	0.2	210 mins
3	0.3	180 mins
4	0.4	6 mins 42 secs
5	0.5	5 mins 54 secs
6	0.6	5 mins 1 sec
7	0.7	4 mins 1 sec
8	0.8	3 mins 28 secs
9	0.9	3 mins 19 secs
10	1.0	3 mins 10 secs

In the following Tables XIII and XIV, some results are given showing the effect of the time interval between addition of taurocholate to the cells and the addition of the serum in causing the observed acceleration of hæmolysis, the experiments have been done in isotonic sucrose solution, which incidentally shows that sucrose solutions behave in an entirely similar manner to that of the saline solution. The concentration of corpuscles is one per cent of which 0.2 c.c. has been used each time. The taurocholate concentration is 0.1 per cent of which one c.c. has been used each time. Other conditions remain the same.

TABLE XIII

Amount of serum in c.c. added $\frac{1}{2}$ minute after the addition of taurocholate	Time of complete hæmolysis
0.0	2 mins 20 secs
0.1	12 mins 24 secs
0.2	30 per cent hæmolysis in 1 hr
0.3	5 per cent hæmolysis in 1 hr
1.0	12 mins 56 secs

TABLE XIV

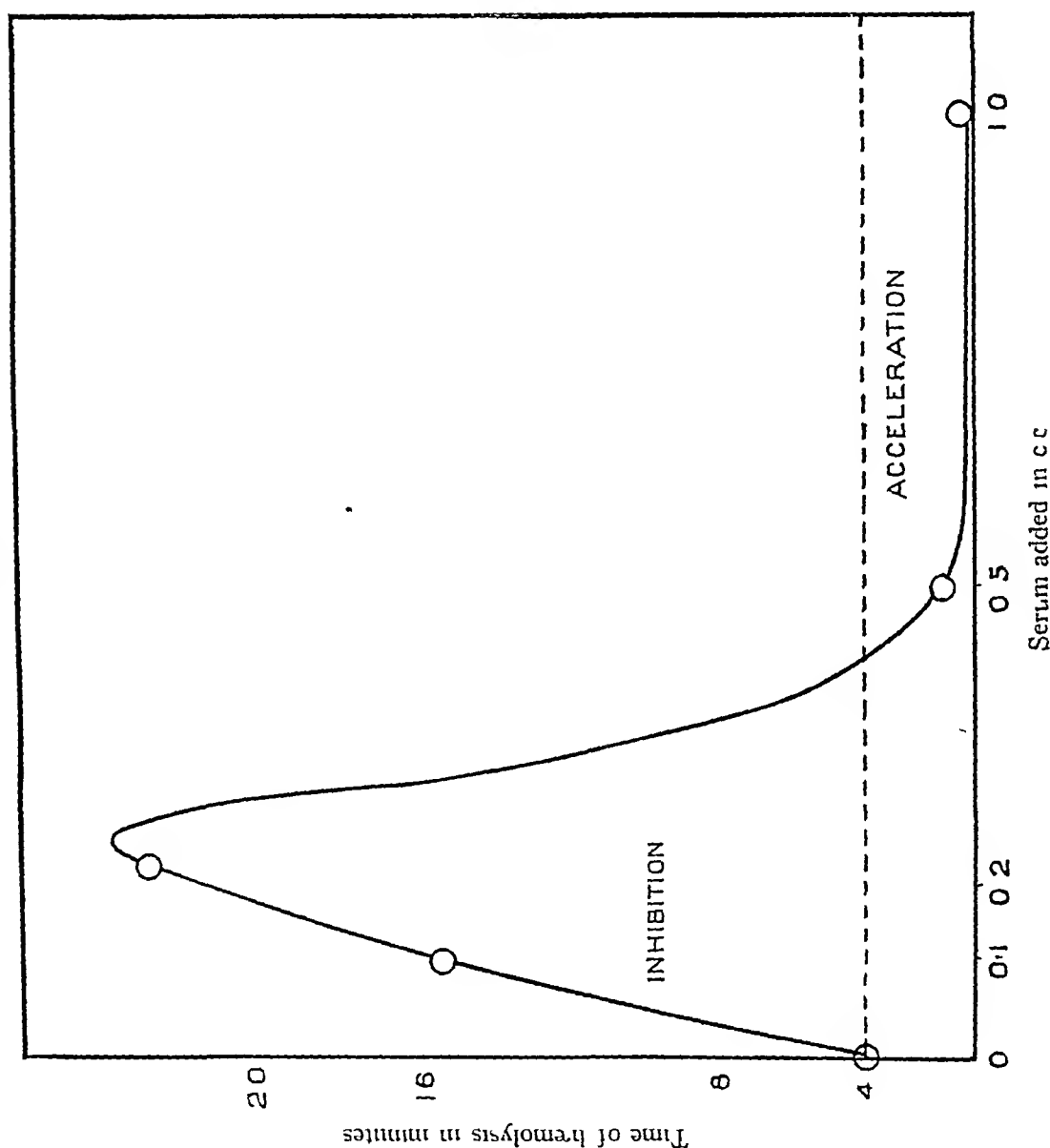
Amount of serum added one minute after the addition of taurocholate	Time of complete hæmolysis
0.0	2 mins 20 secs
0.1	7 mins 4 secs
0.2	2 mins 54 secs
0.3	2 mins 10 secs
0.4	1 min 26 secs
0.5	1 min 3 secs
1.0	Immediate hæmolysis

The data presented in the above tables are very interesting. It will be observed that the addition of normal serum to the red blood cells, before the hæmolyte is actually added, always inhibits the hæmolysis, whereas when serum is added after the addition of the hæmolyte we may get either an inhibition or an acceleration of hæmolysis, depending on the particular concentration of the red blood cells, the hæmolytes, the amount of serum added and in certain cases on the time interval after which the serum is added to the mixture of corpuscles and hæmolyte. Thus it will be observed from Tables VIII, IX and X that with two c.c. corpuscles no acceleration of hæmolysis could be observed when the serum



was added half a minute after the addition of the taurocholate. When 1 c.c. corpuscles was used then also no acceleration of hæmolysis could be observed by the addition of serum when its concentration was less than 0.5 c.c. From Table

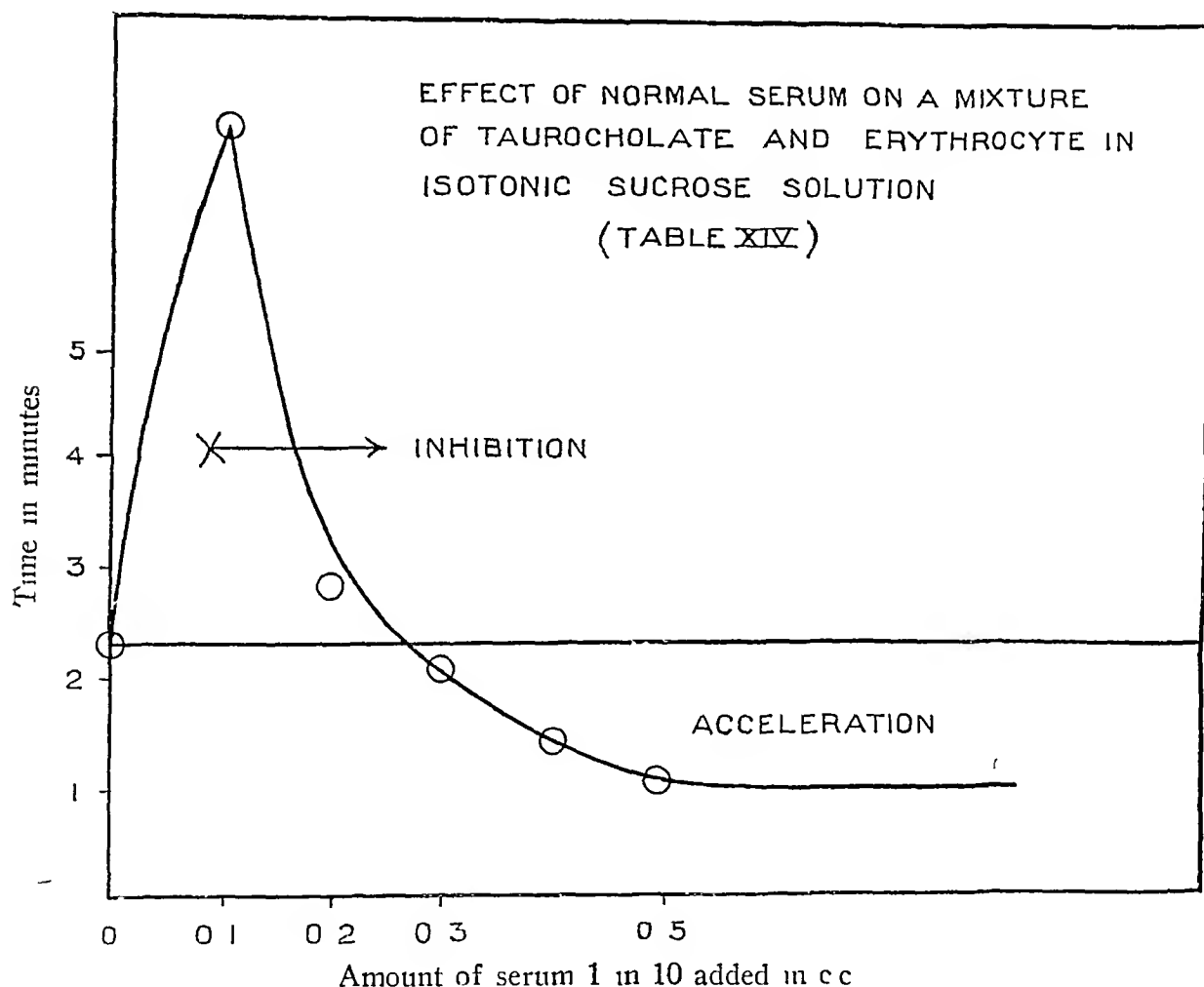
Fig 2



X, with 0.5 c.c. of cells it will be found that we have reached a concentration range in which both inhibition and acceleration of hæmolysis could be obtained depending upon the quantity of serum added. A similar fact is to be noticed in the case of oleate from Tables XI and XII. The results in Tables X and XII

are also very important inasmuch as they allow us to draw a complete and continuous curve of inhibition and acceleration of taurocholate and oleate hæmolysis by the gradual addition of increasing amounts of normal serum to the mixture of corpuscle and hæmolyte after a definite time interval. Two typical curves are given in Figs 2 and 3, and an analysis of these curves will be attempted in a later paper. It will also be observed from Table XIII that even under

Fig 3



similar conditions there was no acceleration of hæmolysis when the normal serum was added half a minute after the addition of taurocholate but when the time interval was one minute as in Table XIV, an acceleration of hæmolysis was observed under identical conditions of concentrations. These facts lead us to the conclusion that the effect of normal serum in hæmolysis is in reality very complex.

#### *V Effect of normally hæmolytic serum in presence of chemical hæmolytes*

While discussing the hæmolytic action of mixtures of hæmolytes, it was stated that an inhibiting action is observed when sodium linoleate is used in

conjunction with a pig serum both of which have a hæmolytic action on sheep's corpuscles. This experiment is of similar nature to that done by Sachs and Altmann (*loc cit*) who found that when sodium oleate was added in proper quantity to a strongly active hæmolytic serum, no hæmolysis resulted, which was supposed to be due to the sodium oleate acting as an anti-complement. We have now made a detailed study of the effect of hæmolytic serum both in active as well as in inactivated condition in presence of other chemical hæmolysers such as saponin, taurocholate, oleate, acids and alkali. For these experiments, human serum containing normally hæmolysins for sheep corpuscles has been used. In the following tables the corpuscle concentration is 1 c.c. of a 0.2 per cent suspension in saline, saponin concentration 1 in 10,000, oleate 1 in 50,000 and taurocholate 0.1 per cent, the total volume being 4 c.c.

TABLE XV

Saponin in c.c.	Time in minutes of hæmolysis in presence of hæmolytic serum, c.c.		
	0.0	0.1	1.0
0.0	—	—	8½
0.8	13½	No hæmolysis in 2 hrs Partial hæmolysis in 2 hrs	—
0.9	11½		—
1.0	9	70	10½
1.1	6½	16	10½
1.2	3	12	10½

TABLE XVI

Taurocholate in c.c.	Time of hæmolysis in presence of hæmolytic serum, c.c.		
	0.0	0.1	1.0
0.0	—	—	8½
0.8	2½	30 per cent hæmolysis in 1½ hrs	—
1.0	1½	80 per cent hæmolysis in 1½ hrs	13½

TABLE XVII

HCl N/500 in c c	Time for haemolysis in presence of haemolytic serum, c c		
	00	01	10
00	—	—	14
08	4	8½	—
10	3	3	70 per cent haemolysis in 1 hr
12	2	2	40 per cent haemolysis in 1 hr

TABLE XVIII

NaOH N/25 in c c	Time of haemolysis in presence of haemolytic serum, c c		
	00	01	10
00	—	—	14
08	4½	8	—
10	3½	6½	11½
12	2½	5	—

So far results with haemolytic serum have been given. In Table XIX, the effects of haemolytic serum and the inactivated serum prepared by heating one sample of the haemolytic serum for half an hour at 56°C, are compared in presence of taurocholate as haemolyte.

TABLE XIX

Taurocholate in c c	Time of haemolysis in presence of 01 c c	
	Haemolytic serum	Complement-free haemolytic serum
15	Slight haemolysis in ½ hr	Slight haemolysis in ½ hr
20	10½	13
25	5	6½

Without serum, the above amounts of taurocholate gave 7 minutes, 3½ minutes and 1½ minutes respectively for the time of haemolysis. The complement-free serum was tested and was found to have no haemolytic power when used alone.

From the results obtained with hæmolytic serum in presence of other chemical hæmolysers, it will be evident that the results of Sachs and Altmann with soaps can be extended to the cases of saponin, taurocholate, acids and alkali. It thus appears that though an hæmolytic serum is capable of hæmolysing red blood corpuscles when present alone, it behaves as an inhibiting agent when added in presence of other chemical hæmolysers. Its qualitative behaviour is almost similar to that of normal serum. But quantitatively its action differs from that of normal serum inasmuch as its inhibiting action is very limited at higher concentrations whereas normal serum at higher concentrations has an almost complete inhibiting action. In comparing the effects of hæmolytic serum and complement-free hæmolytic serum, Table XIX, it will be interesting to note that inactivated hæmolytic serum has a greater retarding action than the other.

#### VI *Hæmolysis in isotonic sucrose*

The results so far given and discussed have been done mainly in physiologically normal saline solution. In a recent paper (Sen and Sen, 1928) it was shown by us that sucrose has an inhibiting action on taurocholate hæmolysis as compared to experiments made in saline solution. Several years ago Ponder and Kennedy (1926) observed an inhibition of saponin hæmolysis in presence of sugar. Ponder and Yeager (1928) have shown the same thing with regard to taurocholate hæmolysis. It has been found by us that sucrose has an inhibiting effect on oleate hæmolysis also. We give below some data about the effect of sucrose on oleate hæmolysis. The concentration of corpuscles is one c.c. of a one per cent suspension.

TABLE XX

Amount of oleate in c.c.	Final concentration	TIME OF COMPLETE HÆMOLYSIS	
		In saline	In sucrose
0.5	1/100,000	13 mins. 50 secs	19 mins 31 secs
0.6	1/83,333	8 mins 1 sec	15 mins 54 secs
0.8	1/62,500	2 mins 45 secs	13 mins 5 secs
1.0	1/50,000	1 min 40 secs	11 mins 2 secs

It will be thus observed that sugars have a definite retarding action on saponin, taurocholate and oleate hæmolysis. Ponder and Yeager in their recent paper (*loc cit*) have shown that the effect of sugars is mainly on the corpuscles themselves, a view already advanced by us previously. It was, therefore, desirable to find out whether hæmolysis in sucrose solutions show an analogous behaviour to that in saline solutions so far as the different aspect of hæmolysis already studied in saline solutions are concerned. To make a full investigation,

therefore, we have made experiments on the nature of the time-dilution curves of taurocholate, saponin and oleate haemolysis with different concentration of blood and the haemolytes effect of normal serum on the acceleration and retardation of haemolysis, etc., in isotonic sucrose solutions. We have already given some data on the effect of normal serum in Tables XIII and XIV which show an identical behaviour to that obtained in saline solutions. An almost analogous behaviour has also been observed in the case of taurocholate, saponin and oleate haemolysis and we have come to the conclusion that haemolysis in sucrose solution shows in all important respects a similar behaviour to that carried in saline solution, the only difference being a depressing action of sucrose on the haemolytic efficiency of different haemolytes by changing the nature of the corpuscle membrane to a certain extent.

### *VII The mechanism of haemolysis*

In the preliminary introduction it was observed that the membranes of the red blood corpuscles are composed of substances which are colloidal in nature and part of them such as the lipoids (lecithin, cholesterol, etc.), can be easily dispersed as hydrophylic and hydrophobic colloids in water. It has already been known from sometime past that a solution of bile salts has a dissolving effect on lecithin and cholesterol. In a previous paper (Sen and Basu, 1928) we have shown that soaps, bile-salts and saponin peptize the lipoids easily, consequently we can assume that the effect of these substances, which also lowers the surface tension of water greatly, in haemolysis is due to a peptization of the lipoidal constituent of the corpuscle membrane. It is of course true that the presence of the protein will have an effect on this peptization. It is well known that corpuscle membranes are slightly negatively charged in saline or sucrose and Gough (1924) has shown that the agglutinating power of different metallic ions in the case of sheep's corpuscles is in the order  $Ce > Th > Ca > K_4 Fe (CN)_6$ . It will be interesting at this place to draw an analogy with an inorganic colloid. Thus we have found that the coagulating power of these cations on a copper ferro-cyanide sol is  $Ce > Th > Ca > K_4 Fe (CN)_6$  beginning with the highest. Copper ferro-cyanide further offers a closer analogy in that it forms membranes with properties very similar to those of the corpuscles membrane.

Thus it is impermeable to sugars and to many substances which is quite analogous to the behaviour shown by the stroma. A recent investigation by Gurchot (1926) has shown that the variable permeability of the copper ferro-cyanide membrane in presence of different substances is caused by the coagulation or the peptization of the membrane and he has drawn attention to the fact that the change in permeability of many plant cells in presence of alcohols investigated by Czapek (1910) and the results of Walden (1893) with different organic acids is nothing but due to a coagulation of the membrane. If now a similar view is advanced to explain the permeability of the stroma, the following becomes obvious. We can consider that ordinarily the corpuscle membrane is in a peptized condition and consists of fine granular particles, the interspaces of

which are filled in by the adsorbed aqueous medium, an assumption exactly analogous to that of the copper ferro-cyanide membrane. Hence water and many water soluble substances can pass through the membrane. Aqueous solution of some neutral salts are, however, not easily permeable because the stroma is polarized owing to the existence of an electrical charge. Salts which have a high coagulating power on the stroma may make the membrane permeable by coagulating it and forming coarse flakes, but at the same time the hæmoglobin may also be precipitated. That with a low concentration of strongly coagulating ions, a permanent change occurs in the stroma is shown by the results of Mikwa (1924) who found that small amounts of uranyl acetate actually damage the structure of the cells as is shown by the increased sensitiveness to physiological saline. The impermeability of the sugars may, however, be due to a different cause, namely due to negative adsorption, a fact well known in the case of copper ferro-cyanide membrane. We can, therefore, summarize the phenomenon of hæmolysis as depending on the following several factors —

- 1 Coagulation of the corpuscle membrane whereby the membrane material will form coarse flakes and hence gives an increase in permeability

- 2 Peptization of some of the membrane constituents such as the lipoids whereby the whole membrane will get loosened and an increase in permeability will occur

- 3 Mechanical or other forms of rupture such as due to swelling or imbibing of water either by the cell as a whole or by any component of the membrane

- 4 Pure solubility effect as for example in the dissolving action of some organic solvents over some of the membrane constituent

These factors will account for the effects of heavy metal salts which at low concentrations act as coagulants, the effect of soaps, saponin, bile-salts, etc., which are good peptizing agent for lipoids, the action of narcotics and of hypotonic and hypertonic solutions. The effect of acid and alkali already studied may also be due to a decrease or increase in the peptizability of the membrane or due to a hydrolytic effect on the lipoids. For a full discussion of this point our previous paper (Sen and Basu, 1928) may be consulted.

There are, however, several minor points which cannot be clearly explained as yet. Thus, it was suggested by us previously that the effect of normal serum in the retardation of hæmolysis by different hæmolytes is due to a displacement of adsorption of the hæmolyte on the corpuscle surface, the serum being preferentially more adsorbed. This view agrees well with the experimental results of sedimentation and agglutination of corpuscles in presence of serum. The accelerating action of normal serum in some conditions as given in this paper cannot however be explained so easily. We do not however believe that a new body of protein + hæmolyte complex having a much greater hæmolytic efficiency is formed when serum is added to a taurocholate + corpuscles or oleate + corpuscles mixtures. The explanation of the acceleration observed when serum is added to a mixture of oleate and corpuscles or taurocholate and corpuscles must

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# SOME OBSERVATIONS ON THE UREA CONCENTRATION TEST OF MACLEAN AND DE WESSELOW

BY

MAJOR S L BHATIA, M C, M A M D (Cantab), M R C P (London), I M S  
J D DUNDAS, M B, B S,

AND

MISS S M COOPER, M B, B S

*(From the Physiological Laboratory, Grant Medical College, Bombay)*

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LABORATORY tests for renal efficiency are obviously based on a knowledge of the normal physiological activity of the kidneys. The functions of the kidneys are to excrete water, certain nitrogenous waste products and many toxic substances that may gain entrance into the body. They regulate the osmotic pressure of the blood by maintaining the optimum concentration of salts in the tissues and body fluids. By removing certain acid products, they maintain the acid-base equilibrium of the blood. They not only remove waste products, but on the other hand are able to prevent the elimination of useful substances, e.g., glucose. Water and chlorides are excreted in large amounts, when present in excess, but their elimination is markedly diminished, when the body lacks them. The work of the kidneys consists chiefly of removal of preformed constituents, for, with the exception of hippuric acid and possibly ammonia, all the other constituents of the urine are formed in parts of the body other than the kidney. Therefore any changes that may occur in the blood plasma are normally reflected in the composition of the urine, and we may say, that the function of the kidney is to keep blood plasma more or less normal in composition. In the formation of the urine, the processes which play a part in varying proportion are filtration, selective absorption and secretion. So far as the excretion of waste products of nitrogenous metabolism, namely urea, uric acid, etc., are concerned, it is to be borne in mind that the kidney is able to concentrate them to a very great extent. Thus, comparing the amounts of urea, uric acid and creatinin in blood and in urine, it is found that the concentration by the kidney is 72, 29 and 40 times respectively. This power of concentration may be even

greater, especially in regard to urea. When the renal cells are diseased, this power to concentrate becomes markedly reduced. Therefore some idea regarding the extent to which the kidney is damaged may be ascertained by an estimation of any of the substances just mentioned. Of all these, urea is easily estimated both in the blood and the urine and a knowledge of the concentrating power of the kidney for urea is the basis of the well-known urea concentration test of Maclean and De Wesselow.

The technique of the test in Maclean's own words is as follows —

'The patient is asked to empty the bladder, and immediately afterwards he receives by mouth 15 grammes urea dissolved in about 100 c.c. of water. The bladder is emptied one hour and two hours after the urea has been given and the specimens of urine examined for urea content. Thus if urea is given at 10 A.M., a specimen of urine is obtained at 11 A.M. and one at 12 noon. If either specimen gives a percentage of urea above 2, the kidneys are held to be fairly efficient, the higher the concentration the more effective is the renal function. The reason why two specimens are taken is that in certain patients the urea given by mouth may produce a diuresis, which tends to dilute the urine passed during the first hour. In this case, the second hour's specimen should be examined. Indeed in routine work, it is generally best to discard the first specimen altogether and to rely on the result obtained from the second specimen. *Not more than about 120 c.c. urine should be passed in the second hour.* Occasionally, if there is much available fluid in the patient's system, it may be necessary to take a specimen after three hours, or even to repeat the test, but this is seldom necessary. In patients with marked diuresis, this must be allowed for in estimating the renal function. In order to avoid the tendency to diuresis as much as possible, it is important that the patient should be warned to take *as little fluid as possible* for 12 to 18 hours before the carrying out of the test.'

The main point is, that in the urine collected 2 hours after the oral administration of 15 grammes of urea, if the percentage of urea is less than 2 the kidneys are held to be less efficient than normal. It is a provocative test. The blood is flooded with urea, and the kidneys are provoked to excrete urea at higher concentrations than on ordinary diet. It has been found, that  $2\frac{1}{2}$  hours after taking 15 grammes of urea, blood urea varies from 40 to 80 mg., while the normal figure on average diet is 15 to 40 mg. per 100 c.c. of blood.

The urea estimations are carried out by the hypo-biomite method. The test is simple. It has been used in a large number of cases, by Maclean and others, and has been found to be very useful in determining the state of the kidneys. It has been claimed that lesions of a much slighter degree can be detected by this means than by estimation of blood urea. The test is perfectly safe and can be applied even to cases showing symptoms of uræmia. To mask the taste, about 1 c.c. of tinct. auranti may be added to the urea solution, but this is not often necessary. It is to be borne in mind, that like other tests of renal efficiency, the urea concentration test gives positive evidence only when the kidney has already suffered gross damage, and that it gives no indication of the

cause of the damage whether it be due to nephritis, calculus, tumour, or some other lesion. But when taken with other clinical findings, it affords very useful information regarding the condition of the kidneys.

Obviously, in order to interpret the test in any given case a knowledge of the normal data is necessary. The object of the present investigation was to ascertain normal standards for Indians owing to possible differences as compared with English figures, depending on different dietetic habits and climate conditions.

The technique followed was precisely the same as advocated by Maclean. Altogether 38 normal persons were examined, of whom 20 were males, 12 females (non-pregnant) and 6 females (pregnant). Investigations in pregnant females were carried out to ascertain if normal pregnancy has any effect on the efficiency of the kidneys. The tests were carried out in the morning before breakfast, no fluid having been taken overnight. Twenty males consisted of 1 medical man, 4 medical students and 15 laboratory servants, and observations on these are recorded in Table I. Twelve females (non-pregnant) consisted of 2 medical women, the rest being all students, and the results of these are recorded in Table II. Table III is a record of observations on 6 normal pregnant women, all from Bai Motlibai Hospital. The age and diet in each case were recorded. The bladder was emptied prior to the administration of urea. This urine is called '*Specimen A*'. It was carefully examined to eliminate abnormal cases. Its volume, specific gravity and reaction were carefully noted. It was examined for albumin. For this, the Salicyl Sulphonic Acid test was applied. The percentage of urea in this sample was estimated. After careful centrifugalization, the sediment was examined microscopically for casts, etc. All cases showing albuminuria, casts, etc., were excluded.

Then 15 grammes of urea dissolved in 100 c.c. of water were given by mouth. Urine was passed after one hour. This is called '*Specimen B*'. Its volume and percentage of urea were carefully observed.

Urine was passed again after 2 hours. This is called '*Specimen C*'. Its volume and percentage of urea were again carefully estimated and recorded. In some cases a third-hour sample was also obtained and analysed for urea content. But this has not been included in the routine observations.

All urea estimations were carried out by the hypo-bromite method described by Maclean.

The results of the observations are tabulated as follows —

Table I Males (20)

Table II Females (12)

Table III Females (pregnant) (6)

Table IV gives the average percentage of urea in Specimens A, B and C as well as the average quantity of urine in the second-hour Specimen C of all the 38 normals including males and females.

It will be observed that —

(1) The average percentage of urea in the second-hour sample is well above 2. Not only the average, but the actual percentage in all the 38 normals, except

## Some Observations on Urea Concentration Test.

TABLE I  
Males

URIA CONCENTRATION TEST															
URINE SPECIMEN A (BEFORE TEST)															
No	Name	Age	Diet M = Mixed V = Veg	Profession	Vol in cc	Sp gr	Reaction	Albumen	Per cent urea	Casts, etc	SPECIMEN B			SPECIMEN C	
											Vol in cc	Per cent urea	Vol in cc	Per cent urea	Vol in cc
1	J D	30	M	Medical man	84	1018	Acid	Nil	2.28	Amorphous phosphates	31.5	3.15	58	2.6	
2	B V	32	M	Lab servant	35	1020	Acid	Nil	1.45	Uric acid crystals	47	2.8	35	3.45	
3	N D	23	M	Lab servant	100	1020	Acid	Nil	1.22	Uric acid crystals	63	2.5	60	2.67	
4	B M	25	M	Lab servant	78	1018	Acid	Nil	0.75	A few amorphous urates	58	1.66	54.5	2.52	
5	D B	30	M	Lab servant	54	1020	Acid	Nil	2.07	Oxalates amorphous urates	50	2.43	35	3.01	
6	B G	19	M	Lab servant	66	1020	Acid	Nil	1.5		49	2.4	36	2.99	
7	K R.	20	M	Lab servant	18		Acid	Nil	1.37	Amorphous urates	31.5	2.56	45	3.1	
8	D G	35	M	Lab servant	77	1012	Acid	Nil	0.95	Amorphous urates	55	2.35	51	3.4	
9	B S	27	M	Lab servant	25		Acid	Nil	2.15	Amorphous urates and uric acid crystals.	29	3.4	50	3.97	

10	D G	45	M	Lab servant	83	1015	Acid	Nil	0.75	Oxalates and amorphous urates	69.5	1.79	45	2.28
11	B G	45	M	Lab servant	33	1018	Acid	Nil	1.5	Oxalates and amorphous urates	59	2.61	64	2.36
12	J G	27	M	Lab servant	66	1014	Acid	Nil	2.0	Oxalates and amorphous urates	57	2.6	54	2.75
13	K M	29	M	Lab servant	24		Acid	Nil	2.3	Amorphous urates	36	3.07	44	3.9
14	M B	40	M	Lab servant	54	1004	Slightly alkaline	Nil	0.21		89	1.2	57	2.15
15	P G H	26	V	Medical student	160	1010	Acid	Nil	0.45	Amorphous urates	64	1.21	69	2.17
16	G K M	22	M	Medical student	436	1010	Acid	Nil	1.07		131	0.77	71	2.015
17	B N P	34	M	Lab servant	112	1030	Acid	Nil	2.63		54	2.4	65	2.3
18	B P D	21	M	Medical student	44	1021	Alkaline	Nil	1.61		84	2.35	45	2.42
19	V R G	23	V	Medical student	42	1021	Acid	Nil	1.62		23	2.2	29	3.08
20	V L	25	M	Lab servant	61	1016	Acid	Nil	1.44	Oxalates and amorphous urates	69.5	2.26	58	2.42

TABLE II  
Female (Non-pregnant)

URINE SPECIMEN A (BEFORE TEST)											URIA CONCENTRATION TEST			
No	Name	Age	Diet M = Mixed V = Veg	Profession	Vol in cc	Sp gr	Reaction	Albumen	Per cent urea	Casts, etc	SPECIMEN B		SPECIMEN C	
											Vol in cc	Per cent urea	Vol in cc	Per cent urea
1	M T	21	M	Medical student	63	1018	Acid	Nil	0.9	A few ovals	96	1.6	68	1.94
2	A B	52	M		250	1010	Acid	Nil	1.8		42	2.3	56	3.07
3	G L	23	V	Medical student	10		Acid	Nil	1.59		61	1.95	50	2.35
4	S K	24	V	Medical student	61	1019	Acid	Nil	1.09		40	1.9	72	2.25
5	S M	23	M	Medical student	33	1017	Acid	Nil	1.97		55	2.45	24	4.75
6	S R	24	M	Medical woman	160	1010	Acid	Nil	1.25		106	2.05	46	2.53
7	I K	23	V	Medical woman	105	1010	Acid	Nil	1		69	2.27	48	3.47
8	M B	37	V		132	1012	Acid	Nil	0.8		45	2.3	32	2.9
9	T C	23	M	Medical student	18	1011	Acid	Nil	1.55		28	2.76	49	3.66
10	D P	37	M			1015	Acid	Nil	0.85		62	2.16	50	2.9
11	S C	7	M	Student	120	1010	Acid	Nil	1.8	Amorphous urates	58	2.75	40	2.51
12	S L	25	V	Medical student	60	1014	Acid	Nil	0.87	Triple phosphates	104	2.6	93	2.62



TABLE IV

No		Average per cent urea. Sample A	Average per cent urea Sample B	Average per cent urea Sample C	Average quantity of urine in cc Sample C
1	20 normal males (Table I)	1.46	2.28	2.77	51.2
2	12 normal females (Table II)	1.29	2.25	2.91	52.3
3	6 normal females (pregnant) (Table III)	1.25	2.41	3.23	57.5
4	Average of all 38 normal persons (including males and females)	1.33	2.31	2.97	53.6

No. 1 in Table II is higher than 2. The highest figure for urea concentration is 4.75 per cent (Case 5, Table II). This shows that the power of the kidneys to concentrate urea is as good in the Indian as in the Englishman. No marked variation is observed.

(2) The percentage of urea in Sample A (urine before test) is relatively low, the average being 1.33 per cent and the lowest figure is actually 0.21 per cent. This is, no doubt, due to the low protein diet of the Indian. We have tried to classify these 38 normals into vegetarians and non-vegetarians. According to their own statements, 7 were pure vegetarians and 31 took mixed diet of meat and vegetables. But the amount of meat taken per day even by those who are not professed vegetarians is small, much smaller than the daily meat ration of Englishmen in England. Hence, the urea content of the urine of the Indian is generally lower than that of the Englishman. The average figure for Englishmen, or those who live on a generous ration of meat, is 2 per cent or higher.

We think in order to adjudicate the results of the urea concentration test, a knowledge of the percentage of urea in Sample A is essential. If the urea percentage in this sample is low, then concentration up to 2 after 2 hours denotes efficient kidneys. Thus Case 1 in Table II had 0.9 per cent urea in the Sample A, while Sample C contained 1.94 per cent. This shows that the concentrating power of the kidney is quite good in spite of the figure being just below 2.

From the figures available, there does not appear to be any real difference between the vegetarian and non-vegetarians as regards their power to concentrate urea.

(3) The volume of the second-hour Sample C is larger in the case of Englishmen than in Indians. For 38 normal Indians this volume is on the average 53.6 cc., while for Englishmen as worked out from Maclean's table for persons



of the same age-groups it is 100.2 c.c. Further in no case did the amount exceed 120 c.c. It is to be observed that there is less tendency to diuresis after administration of urea in Indians than in Englishmen.

(4) When reckoned according to age, it is to be observed, that of the 38 normals, 1 is below the age of 18, 4 between 19 and 25, 32 between 26 and 45 and one between 46 and 65 years. Thus a large majority of these belong to the adult age-group. We have not yet sufficient figures for the other age-groups, but there is no doubt, as stated by Maclean, that as age advances the power to concentrate urea diminishes.

(5) Table III shows that normal pregnancy does not effect the efficiency of the kidneys and that the urea concentrating power of the kidneys, when tested, is well within normal limits.

Recently Stott and Mangalik have published standards for this test in healthy Indians. They also conclude that there is no difference in the power of urea concentration either at all ages or in the different age groups between healthy kidney of normal Indian and normal Englishman in spite of marked dietetic differences. They also observed, as we did, that the Indians secreted less urine during the second hour than Englishmen.

Now, this test has been applied in a large number of hospital cases in this laboratory. The cases were both medical and surgical and came from the J J Hospital. There is no doubt, that the test is of great value in determining the efficiency of the kidneys. By way of illustration, we may quote the data obtained from 5 cases of chronic nephritis showing low urea concentration. The results are given in the following table —

TABLE V (*Medical Cases*)  
UREA CONCENTRATION TEST

No	Name	URINE SPECIMEN A	SPECIMEN B		SPECIMEN C		REMARKS
		Per cent urea	Vol in c.c.	Per cent urea	Vol in c.c.	Per cent urea	
1	M G	0.6	130	1.17	87	1.57	No albuminuria
2	F S V	1.125	114	1.34	106	1.68	No albuminuria. Granular and epithelial casts present
3	V P	0.78	56	1	107	1.15	Third-hour sample Vol 50 c.c. Urea 12 per cent. Albuminuria present
4	P D S	0.35	57.5	0.8	49	1.08	Albuminuria and granular casts present
5	T F	0.44	94	0.83	76	1.76	Albuminuria and granular casts present

It will be observed that the urea percentage in either of the samples collected after administration of urea is less than 2, indicating thereby that the kidneys are less efficient. The percentage of urea in Sample A is also low, much lower than the usual figure of 2 per cent given for Europeans, but not so low as compared with the figures we have just put forward for 38 normals. It is clear that no conclusions whatever can be drawn from a single estimation of urea in the urine. The only way in which these estimations do help is by administering urea by mouth and so flooding the kidney by excess of urea in the blood. If then the percentage rises to 2 or over, the kidneys may be considered to be quite good.

In dealing with medical cases the test is specially helpful in Chronic Interstitial or Azotæmic Nephritis, in which œdema is slight or absent, albuminuria is also slight or absent, the chloride content of the urine is normal, there is tendency to increase of urea and other nitrogenous waste products in the blood, marked cardiovascular changes, and tendency to uræmia. In such cases urea concentration is low, the figures being below 2 per cent. In Hydræmic or Parenchymatous Nephritis with œdema, marked albuminuria, absence of cardiovascular changes and less tendency to uræmia, the urea concentration gives normal figures. But in this case, Nature has herself carried out an efficiency test, for the presence of œdema in association with retention of chlorides is a visible demonstration of the fact that the kidneys are not excreting salt. In later stages, Parenchymatous Nephritis may pass into Azotæmic or Interstitial Nephritis. Then this test gives evidence of the diminished power of the kidney to concentrate urea.

The physician often needs information regarding the efficiency of the kidneys in affections other than Nephritis, in which the kidneys may be involved. In such instances the test is very helpful. There is one interesting observation in our records, which shows that during an attack of malaria, the efficiency of the kidneys is impaired. L. V., a laboratory servant aged 25, was prepared for the urea concentration test. During the test, he was suffering from an attack of malaria. His blood was examined and malaria parasites were found (both B 'T' and M 'T'). The urea concentration test gave the following result —

	Per cent urea	Volume in c.c.
Urine Sample A	1.55	130
„ „ B	0.875	200
„ „ C	1.695	82
„ „ D	1.8	79

Sample D = third-hour sample

He was put on anti-malarial treatment, and the urea concentration test was again done 3 weeks later with the following result —

			Per cent urea.	Volume in c.c.
Urine	Sample	A	1.44	61
"	"	B	2.26	69.5
"	"	C	2.42	58
"	"	D	2.42	56

There was no albuminuria at any stage. It shows that the kidneys become less efficient during an acute malarial attack, but this is a temporary effect as recovery soon occurs under appropriate treatment.

This test does not tell us what the cause of the renal inefficiency is. No renal test does this. It, however, does help considerably in forming our judgment regarding the existing condition and the future outlook of the patient.

It is also helpful in estimating the renal function in certain surgical affections of the genito-urinary tract, e.g., enlarged prostate. Whenever there is long continued obstruction to the passage of the urine, the kidney is involved sooner or later and its efficiency is diminished. Under these circumstances a major operation may be disastrous and should be postponed, nothing more than the most urgent surgical procedure should be carried out. Thus in enlarged prostate, suprapubic cystotomy only should be carried out at first. The major operation of prostatectomy can be carried out with comparative safety at a later stage. The kidneys recover to a large extent when the obstruction is removed. The renal damage in such cases does not appear to be of a permanent nature. In such cases a combination of the urea concentration test and estimation of blood urea gives valuable help. If these are normal, the operation can be carried out with relative safety. The following case may be cited by way of illustration —

D I C, aged 55 J J Hospital. Suffering from enlarged prostate.

Urea concentration test

			Per cent urea	Volume in c.c.
Sample	A		2.51	17
"	B		2.39	40
"	C		2.66	25
"	D		2.72	33

Prostatectomy was done and the patient made a good recovery.

In surgical cases additional help may be obtained by applying one of the dye tests, namely, indigo carmine or the phenol sulphon phthalein test, and by

ureteric catheterization the lesion may be localized in one or the other kidney. Finally, as Harrison says, the urea concentration test being a provocative test, when applied to damaged kidneys, it makes a call on some of their 'reserve,' and for this reason it is possibly a more sensitive test than many others, e.g., estimation of blood urea. Some idea of the 'reserves' of the kidney may be had from the fact that a man with one kidney excised may give a normal urea concentration test. This will be illustrated by the following case —

C. T., Hindu, aged 23. J. J. Hospital. Suffering from a cystic swelling in left lumbar region and left iliac fossa for 5 years, which is dull and painful on pressure. It had been tapped thrice. The last tapping was 4 days before admission. History of hæmaturia for 48 hours. Diagnosis: Hydronephrosis (left). Urine alkaline, abundance of red blood corpuscles, deposit showed phosphates.

Urea concentration test

	Per cent urea	Volume in c.c.
Sample A	2.26	73
" B	3.34	36
" C	3.58	45

Blood urea = 31 mg per 100 c.c. of blood

*Operation*—Left kidney was removed. Urea concentration test 3 weeks after the operation —

	Per cent urea	Volume in c.c.
Sample A	1.1	77
" B	1.52	103
" C	2.74	39

Thus it will be seen that the urea concentration test gave evidence of good kidneys in spite of the fact that the total amount of renal tissue was reduced by half. It is to be borne in mind that two-thirds or more of the total renal tissue may be damaged before this or any other test for renal efficiency gives positive information.

### CONCLUSIONS

1. Normal standards for urea concentration test of Maclean and De Wesselow based on 38 normal subjects are given. The efficiency of the kidney of Indians to concentrate urea appears to be as good as that of Englishmen from whom Maclean obtained his data.

2 Six of the 38 normal subjects were pregnant women. The test showed that normal pregnancy does not in any way impair the efficiency of the kidneys.

3 There is less tendency to diuresis after administration of urea in Indians than in Englishmen.

4 The average percentage of urea in urine of Indians is lower than that of Europeans, owing possibly to smaller amount of protein in the diet.

5 The value of the test is discussed as well as its application to certain Medical and Surgical ailments.

We are much indebted to the Physicians and Surgeons at the J J and Bai Motilal Hospitals for their help and co-operation.

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| <i>Idem</i> (1928)                   | <i>Ibid</i> , p. 1143  |
| MACLEAN, H. (1924)                   | 'Modern Methods in the Diagnosis and Treatment of Renal Disease' |
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# THE ANATOMY OF THE INDIAN *XENOPSYLLA* LARVÆ

BY

MAJOR W J WEBSTER, M C, I M S,  
Haffkine Institute, Bombay

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THE classical description of the rat-flea larvæ by Bacot and Ridewood(1) includes a full account of the larvæ of *Xenopsylla cheopis*. No record of the anatomy of the larvæ of *X. astia* or *X. brasiliensis* has been traced in the literature available. Some additional notes on *cheopis* larvæ have been published recently by Henderson(2), whose material consisted of a collection of larvæ sent from the Parel Laboratory to Edinburgh, and it has been assumed that these are *cheopis* larvæ. The collection was sent in 1909, and at that date all non-pectinate rat-fleas in Bombay were classed as *cheopis*. It has since been accepted that the three species are represented (Table I), and it would appear unlikely that the larvæ referred to are *cheopis* only. Although *cheopis* is about four times as numerous

TABLE I  
*Fleas collected from the rat-pit, Haffkine Institute*

1928	<i>X. cheopis</i>			<i>X. astia</i>			<i>X. brasiliensis</i>			Others	Total
	♀	♂	Total	♀	♂	Total	♀	♂	Total		
August	244	330	574	73	43	116	8	6	14	1 <i>Echidnophaga</i> ♂	705
September	77	124	201	22	16	38	2	2	4		243
October	92	133	225	35	34	69	5	5	10		304
November	45	101	146	33	19	52	9	7	16	1 <i>Ctenocephalus</i> ♂	215
December	52	52	104	24	10	34	3	3	6		144
TOTAL	510	740	1,250	187	122	309	27	23	50		1,611
PER CENT	31.6	45.9	77.5	11.6	7.6	19.2	1.7	1.4	3.1	0.1	100

as *astia* on Bombay rats, yet when flea eggs are collected by keeping individual fleas in test-tubes, the majority of the eggs obtained are from *astia* females. It, therefore, seemed advisable to examine a number of all stages of the larvæ of the three species in question.

It may be stated at once that the description of the larvæ of *X. cheopis* given by Bacot and Ridewood applies equally well to *astia* and *brasiliensis*. In particular it may be noted that the egg-breaker is present in the first stage larvæ of *astia* and *brasiliensis*, that the maxillary palps are of type A (Plate VIII, fig. 3) and the processes at the base of the antennæ are of type A (Plate VIII, fig. 4) throughout all stages of the larvæ of the three species. In accordance with this, the salivary glands are bi-lobed, thus conforming to the association of certain peculiarities of external and internal anatomy pointed out by these authors. The number and arrangement of the setæ is also identical, and the mandibles are very similar, showing five (occasionally four), teeth occupying the upper inner edge (Plate VIII, fig. 5).

With regard to Henderson's description, it is noted that the rows of small setæ on the anal mounds are much the same in the three species, but that there is considerable individual variation. There are two rows of about eight setæ, with one or more additional setæ at the outer side of the upper end of the rows. The majority of *cheopis* larvæ show three or four of these external setæ. *astia* and *brasiliensis* generally show only one. The anal comb contains from 17 to 24 setæ, the commonest number being 20. The area behind the anal comb has the appearance of a distinct segment, and bears a small lateral seta similar to that on the tenth segment.

It has not, therefore, been found possible to distinguish the three species in the larval stages.

In certain respects the appearances seen are considered to differ from the descriptions previously given.

As regards the tracheal system, Bacot and Ridewood refer to the diagram of a flea larva given by Laboulbène which they consider correct, except that it shows a stigma on the third thoracic segment. Henderson also states that there is no spiracle on the second or third thoracic segments. The position of the opening on the first segment given by Henderson is correct, viz. posterior to both rows of setæ, and not accompanied by any irregularity in the line of the small setæ. There is no opening on the second thoracic segment. On the third thoracic segment, however, a pair of stigmata is constantly present, at all stages of the larvæ of the three species. They are situated immediately in front of the row of small setæ and are not accompanied by any irregularity in the line of setæ (Plate VIII, fig. 1). In specimens mounted flat on the ventral surface, these stigmata are almost on the 'edge' of the body and therefore readily overlooked. In the thorax, the tracheal branch runs almost directly to the surface from the middle of the tube which links the upper and lower longitudinal trunks of the tracheal system. In the abdomen, the branch passes backwards some distance to reach the orifice (Plate VIII, fig. 2). The position of the abdominal stigmata is as described by Henderson.

The processes at the base of the antennæ are quite constant throughout all stages of the three species, viz., three large and three small processes, arranged in a semicircle on the upper outer side of the antenna. In all but the first stage larvæ, a 'scar' suggests an additional, immature process on the inner side (Plate VIII, fig 4)

According to Henderson, the small setæ are absent from the thorax in the first stage larvæ, but although they may be very minute they are constantly present (Plate VIII, fig 1). It may be noted that the rows of large and small setæ on each segment are closely approximated on the ventral surface but widely separated on the dorsum.

Some relevant dimensions of flea-eggs and larvæ are given in Table II, for comparison with the figures given by Bacot and Ridewood. Table III shows the number of small and large setæ on the various segments.

As regards technique, the use of Berlese's fluid [which Fletcher(3) recommends for the examination of mites] has been found satisfactory and time saving. Drowned in 70 per cent alcohol and mounted in this medium, flea larvæ make excellent specimens. If the tracheal system is to be investigated the larvæ should be examined the same day. As the medium is water-soluble, and the specimen is liable to be damaged if allowed to get wet, it is advisable to ring the cover-slip with gold size after a week or two. This mountant has many other applications, e.g., it would appear to be useful for mounting sandflies, entire or dissected, as it clears satisfactorily, and provides a permanent preparation, if required, without the necessity of further manipulation.

### SUMMARY

The larvæ of the three common non-pectinate rat-fleas of India are found to be indistinguishable on anatomical grounds.

Dr Chitre has carried out the major part of the flea-counts.

TABLE II

Species	DIMENSIONS OF FLEA-EGGS		LENGTH OF LARVÆ	
	Length mm	Breadth mm	New born mm	Full grown mm
<i>X cheopis</i>	0.46	0.31	1.4	3.6
<i>X astia</i>	0.44	0.30	1.6	3.2
<i>X brasiliensis</i>	0.42	0.30	1.25	3.3

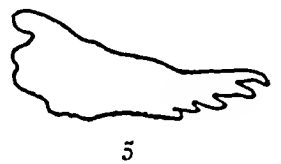
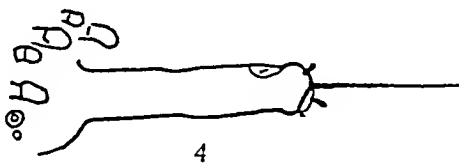
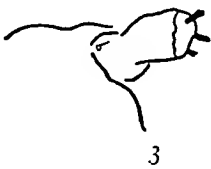
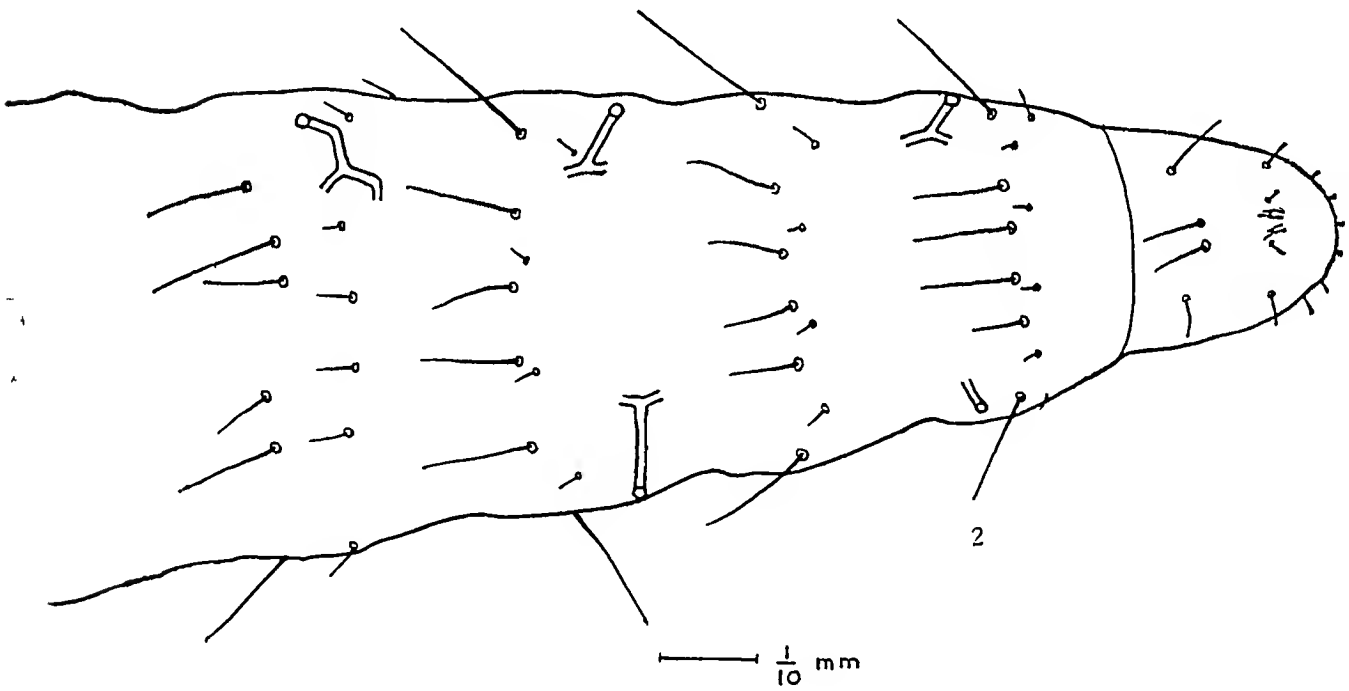
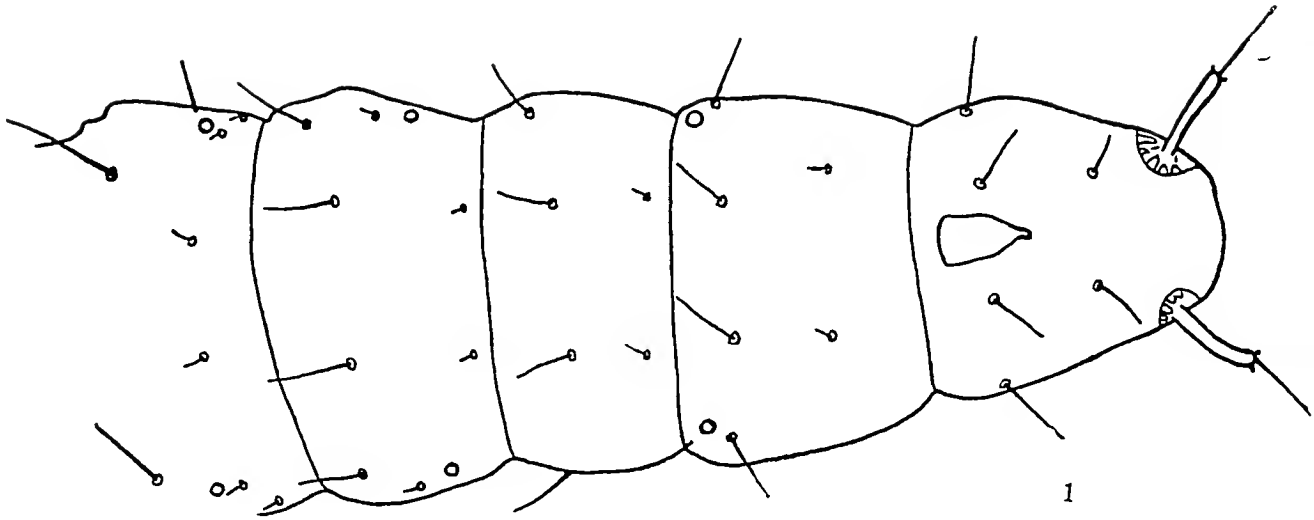
Average of 12 specimens in each case

There is considerable individual variation in the case of the larvæ





# PLATE VIII



$\frac{1}{10}$  mm

TABLE III

*Pairs of setæ on segments of larvæ of X cheopis, X astia and X brasiliensis*

Segments	1-3 T	1-6 A	7 A	8 A	9 A	10 A
Large setæ	5	5	6	6	7	3
Small setæ	5	6	6	6	5	1

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- (1) BACOR and RIDGEWOOD (1914) *Parasitology* VII, 157  
 (2) HENDERSON (1928) *Ibid* XX, 115  
 (3) FLETCHER (1928) *Trans Roy Soc Trop Med and Hyg*, XXII, 2, 161

## EXPLANATION OF PLATE VIII

- Fig 1 Part of newly hatched larva of *X brasiliensis*, showing small setæ on thorax and stigmata on first and third thoracic segments (The antennal processes are not in focus *see* Fig 4)  
 „ 2 Part of full-grown larva *X astia* from ventral aspect to show thoracic stigmata and part of tracheal system  
 „ 3 Maxillary palp of larva *X brasiliensis*  
 „ 4 Antenna and processes of larva *X brasiliensis*  
 „ 5 Outline of mandible of larva *X cheopis*

# OBSERVATIONS ON THE EXCRETION OF ANTIMONY IN THE URINE

BY

LIEUT-COL T C BOYD, FRCSI, DPH, FIC, IMS,  
*Chemical Examiner to the Government of Bengal,*

AND

A C ROY, MSc,  
*Biochemical Research Worker under the Indian Research Fund Association*

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It appeared to us to be of practical importance to gain some insight into the question of the excretion of antimony when administered in the pentavalent and trivalent condition to patients suffering from kala-azar. We selected antimony compound No 693, which is a diethylamine salt of p-aminophenyl stibinic acid as a type of a pentavalent antimony compound and sodium antimony tartrate as a trivalent compound.

In our selection of a method for the estimation of the antimony, we were confronted with some difficulty. The methods commonly employed for the estimation of antimony in the presence of organic matter consist in general principle of —

(1) The deposition of the antimony on a copper foil when boiled in the presence of concentrated hydrochloric acid (Reinsch's method)

(2) Dissolving the deposited antimony in some suitable solvent such as alkaline permanganate (Goldsbrough, Beam and Freak) or alkaline persulphate (Brahmachari, Das and Sen)

(3) Removal of impurities such as iron, copper, etc

(4) Determination of the antimony colorimetrically as sulphide or by titration with iodine

We tried Beam and Freak's modification of Goldsbrough's method but failed to obtain satisfactory results. We next tried Brahmachari's modification of the above methods but with no more success. Our failure to obtain concordant results with this latter method may be due to Brahmachari's omission to give the necessary technical details such as the correct strength of the persulphate solution,

its degree of alkalinity and the time during which the copper foil with the deposited antimony should be boiled with the alkaline persulphate solution. A knowledge of these points is in our opinion essential.

After repeated trials and many failures we found the following method which is in principle a combination of Fresenius and V Babo's method for the destruction of organic matter and Riegel and Sanger's method for the estimation of antimony, the most reliable and expeditious from the practical standpoint.

#### *Description of the method*

The urine is measured and then evaporated almost to dryness on a water bath in a porcelain evaporating basin. Great care is necessary in transferring the urine from the collecting bottles to the evaporating dish as the urine though preserved with formalin undergoes some degree of decomposition with precipitation of antimony and unless the bottle is carefully washed and the washings added to the bulk of the urine serious error may arise.

#### *Destruction of the organic matter*

The evaporated urine is carefully transferred to a 500 c.c. Kjeldahl flask by the addition of successive small amounts of water, about 5 c.c. of concentrated hydrochloric acid (Merck's arsenic free Sp. Gr. 1.124—1.126) and about one gramme of potassium chlorate (Merck's) are added, the flask is then heated over a small flame with frequent gentle agitation of the contents. If within an hour the contents of the flask clears up and longer heating produces no change, the next step in the process may be proceeded with. If not, as is usually the case where one has to deal with 200 c.c. or more of urine, 0.25 to 1 gramme potassium chlorate and 1 to 4 c.c. of concentrated hydrochloric acid are added at frequent intervals until the liquid in the flask assumes a yellowish colour and which does not darken on continued heating. This procedure removes the bulk of the organic matter.

#### *Removal of chlorine*

After the destruction of the organic matter as described 5 c.c. of concentrated hydrochloric acid and sufficient distilled water to make the volume to about 250 c.c. are added and the liquid after the addition of a few glass beads is boiled briskly until the contents reach about 100 c.c. It is then allowed to cool and again 5 c.c. of concentrated hydrochloric acid and distilled water to about 250 c.c. are added and the liquid boiled as before until the bulk is concentrated to about 50 c.c. It is then tested for the presence of free chlorine, either by smell or by means of starch iodide paper. We have found from experience that two boilings with concentrated hydrochloric acid seldom leave any free chlorine in the liquid. It is essential that when boiling to drive out chlorine, the liquid be kept strongly acidified with hydrochloric acid as described, otherwise a big loss of antimony may occur. The presence of the hydrochloric acid in excess serves in some way to prevent this loss and we wish to express our

indebtedness to Mr. R M Roy, msc, for his valuable help in solving this difficulty Our failure with this method in the beginning was due to our lack of knowledge on this important point

During this process it often happens that the solution assumes a brown colour which is due to some undecomposed organic matter, but we have not found this to affect our results

The liquid is now filtered through a Whatman filter paper into a 100 cc measuring flask, the Kjeldahl flask and the filter paper being carefully washed with successive small amounts of hot water and the volume made up to 100 cc The solution is now ready for estimation of the antimony

### *Estimation*

For this we followed in all essentials Sanger and Riegel's adaptation of Gutzeit's method for arsenic estimation to antimony with some minor modifications as to details We were unable to use any method based on titration with iodine such as that of Knorr, as a certain amount of organic matter always escapes decomposition and the presence of even a trace of this vitiates the result We found, however, that the presence of some undecomposed organic matter does not materially affect the result when the Gutzeit's method is employed

### *Procedure adopted*

#### (1) Preparation of the sensitized paper —

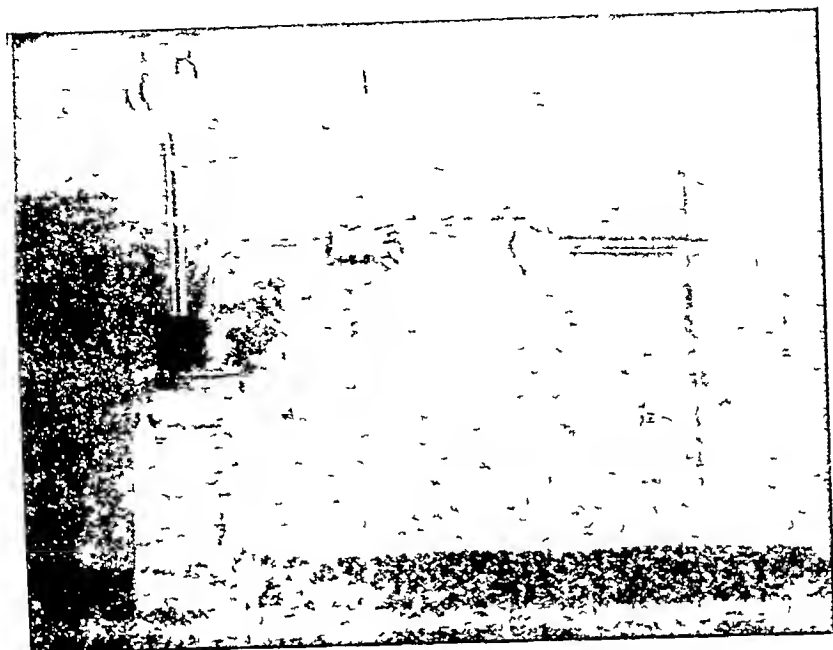
On a Whatman's filter paper, about 18 cm in diameter, strips are marked with a graphite pencil each measuring exactly 5 mm wide by 7 cm long The marked filter paper is then soaked in a 5 per cent solution of mercuric chloride for fifteen minutes in a flat dish and, after removal, drained and dried in a steam oven When dry the paper is carefully cut along the pencil marks to give strips 5 mm by 7 cm The prepared strips must be stored over calcium chloride in a dark place It is essential that great care should be exercised in the cutting of these strips to exact size

#### (2) Apparatus —

This consisted of a wide-mouthed bottle of about 100 cc capacity fitted with a rubber cork through which pass a thistle funnel and an exit tube bent at right angles The latter was fitted with a small rubber cork to which was attached the deposition tube The actual arrangement of the apparatus is shown in the photograph

About nine grammes of uniformly granulated zinc (Merck's arsenic free) is placed on the bottom of the bottle and evenly distributed, the cork replaced and the thistle funnel adjusted so as to extend nearly to the bottom of the bottle A disc of lead acetate paper moistened with a drop of water is inserted in the enlargement of the deposition tube as a precaution against the possible evolution of sulphuretted hydrogen Finally, a strip of mercuric chloride paper is placed in position, care being taken to see that the paper does not present an uneven

surface to the issuing gas. The bore of the deposition tube should be just large enough for the paper strip to slip in.



### *Preparation of the standard bands of antimony*

3.2469 grammes of sodium antimony tartrate is dissolved in 250 c.c. of distilled water. An aliquot part of this solution is titrated against N/10 iodine solution and the antimony content determined. One c.c. of this solution was found equivalent to 4.92 milligrams of antimony (theoretical 5 mg). This solution is labelled antimony solution No. 1. A dilute antimony solution is also prepared by diluting 5.08 c.c. of solution No. 1 to 250 c.c. and labelled antimony solution No. 2. One c.c. of solution No. 2 equals 0.1 mg of antimony. To the apparatus arranged in the manner described a small aliquot part of antimony solution No. 2, say one c.c., is added through the thistle funnel and sufficient hydrochloric acid (1 in 6), to make the total volume to 60 c.c. The reaction is then allowed to proceed for one hour when the paper strip is removed and placed in a small test tube containing approximately normal ammonia solution. A black stain immediately appears but the paper should be allowed to develop for five minutes. It is then washed with distilled water and dried. The stain formed is equivalent to 0.1 mg of antimony. In a similar manner stains are prepared representing amounts of antimony from 0.02 mg to 0.3 mg. For amounts representing less than 0.02 mg or more than 0.3 mg, the stains obtained do not represent the antimony actually present. It is advisable to perform a blank experiment to be certain that the reagents employed do not produce a stain.

To determine the amount present in the unknown solution, an aliquot part is taken and washed through the thistle funnel with hydrochloric acid (1 in 6) to make the volume up to 60 c.c. and the stain obtained compared against the standard stains. If the stain does not fall within the set of standards, larger or smaller quantities as the case may be of the unknown solution must be taken and the experiment repeated. We consider that this method, when carefully followed, yields results correct within 10 per cent.

The following points should be particularly noted —

(1) Each worker must prepare his own standard bands and he also must use apparatus having the same approximate dimensions.

(2) The quantity of zinc should be very nearly the same and the granules as far as possible uniform.

(3) The total volume of the liquid (acid and antimony solution) inside the reaction bottle must be the same in every experiment.

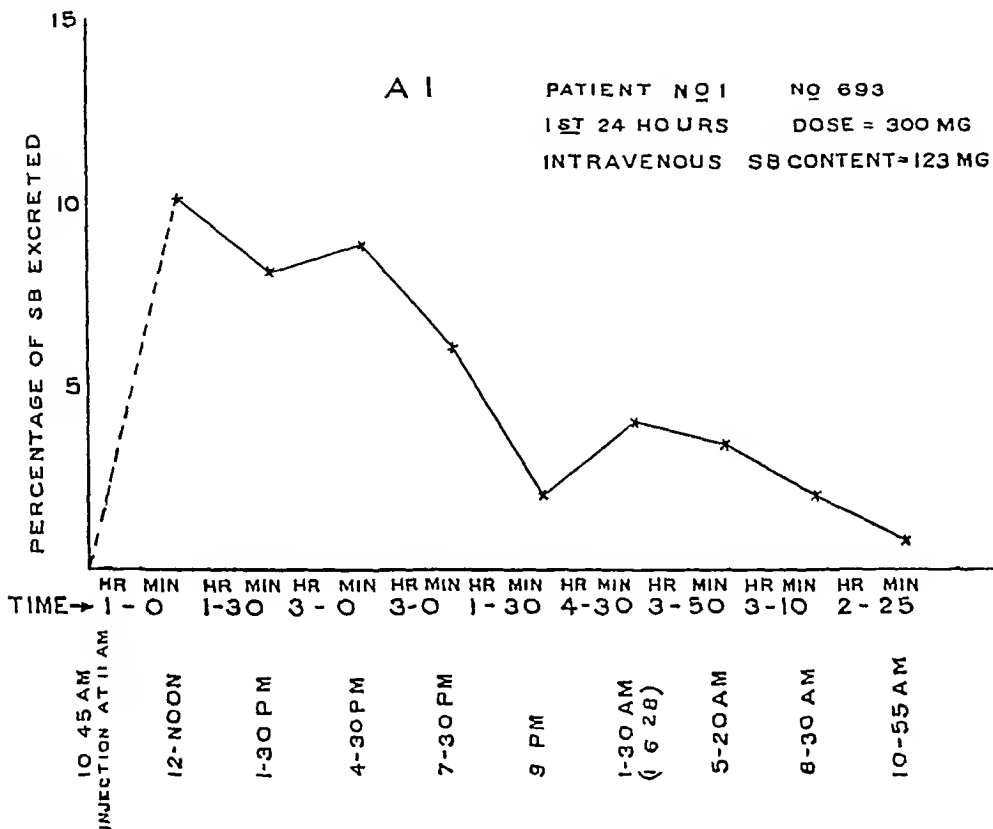
(4) On general principles it might be supposed that the amount of the stain might be influenced by the rate of evolution of the gas and that it would therefore be desirable to have the concentration of acid inside the reaction bottle the same for each determination. In practice, however, we found from a number of experiments in which varied quantities of urine containing known amounts of antimony were used and which required different amounts of hydrochloric acid for digestion that the stains obtained were not appreciably different from the standard bands for those respective amounts.

Utilizing the method as described, we proceeded to study the excretion of antimony in the urine. Two patients were each given a single intravenous injection of compound No. 693 consisting of 300 mg. and representing 123 mg. of antimony. The amount of antimony present in the urine was determined at frequent intervals for the first three days and then for several days in the twenty-four hours collection. The results obtained are shown in graph forms and placed side by side to facilitate comparison. Graphs marked  $A_1$  and  $B_1$  represent the excretion rate in both patients during the first day,  $A_2$  and  $B_2$  for the second day,  $A_3$  and  $B_3$  for the third day, while  $A_4$  and  $B_4$  represent the antimony excreted in every twenty-four hours collectively. It may appear from a comparison of the Graphs  $A_1$ ,  $A_2$ ,  $A_3$  with  $B_1$ ,  $B_2$ ,  $B_3$  that their similarity is not exact. If, however, the amount of antimony excreted for the same period is compared, it will be found that there is a close resemblance, for example in  $A_1$ , 18 per cent of the antimony injected is excreted in two hours and thirty minutes, while in  $B_1$ , 20 per cent is excreted in two hours forty-five minutes. This similarity is better brought out, therefore, in Graphs  $A_4$  and  $B_4$  where it may be noted that about 45 per cent of the antimony is excreted in the first twenty-four hours in  $A_4$  against 38 per cent in  $B_4$ . During the second twenty-four hours, 5 per cent in  $A_4$  and 7.5 per cent in  $B_4$ . During the third twenty-four hours, 1 per cent in  $A_4$  and 1.5 per cent in  $B_4$ . After the third day, the antimony excreted assumes a very low and almost constant value. On an average



it may be stated therefore that about 49 per cent of the antimony is excreted in the first three days

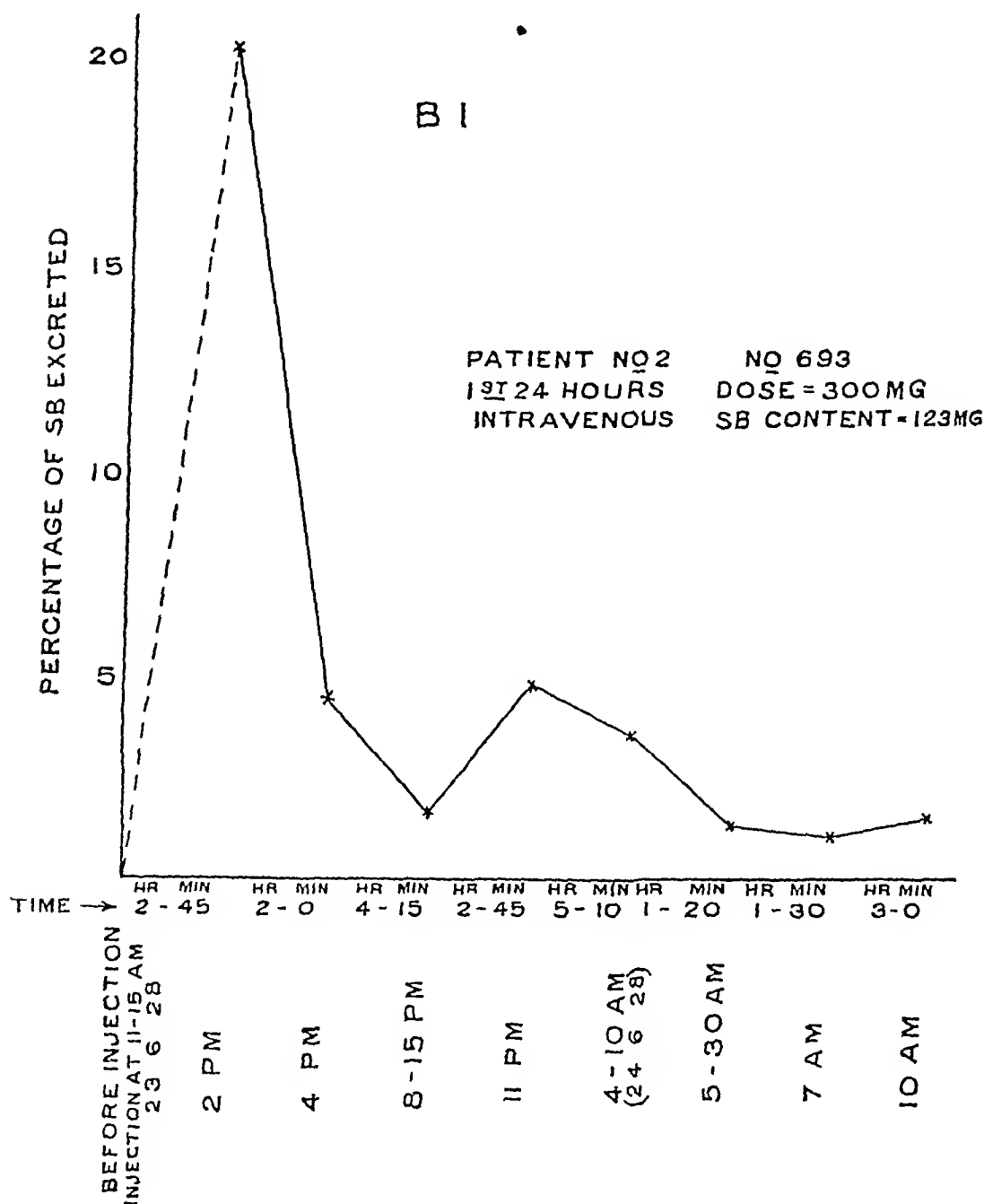
Our next series of observations was concerned with the rate of excretion of the same compound (No 693) when administered intramuscularly. Two patients were each given 300 mg of 693 intramuscularly and the antimony excretion studied on exactly similar lines



The results obtained are given in graph form

It will be noted that the same general agreement which marked the graphs representing excretion under intravenous injections is also present in the intramuscular cases. On comparing Graph C<sub>1</sub> with D<sub>1</sub> it will be seen that about 36 per cent of the antimony is excreted in the first twenty-four hours (C<sub>1</sub>) against 32 per cent in D<sub>1</sub> during the same period. In the second twenty-four hours about 4 per cent against 2 and in the third twenty-four hours 2 per cent against 1. On an average, therefore, about 34 per cent of the antimony is excreted during the first twenty-four hours, about 3 per cent in the second, and 1.5 per cent in the third with an average excretion of about 38 per cent during the first three days. After the third day, the antimony excretion is very low and almost constant. In general terms it may, therefore, be stated that the type of excretion curves in the intravenous and intramuscular administration is

similar with compound No 693, but that there is a perceptible lag in the excretion rate when the intramuscular method is used



The next point that we proceeded to investigate was whether an alteration in the dose of the compound No 693 would affect the picture of the excretion rate. To determine this, a patient was given an intravenous injection of 150 mg of No 693 (half the former dose employed) and the antimony excreted estimated in every twenty-four hours sample. The results are shown in Graph E.

T C Boyd and A C Roy

Percentage of Sb excreted

INJECTION AT 5-20 PM  
(10 8 28)

8-40 PM

5-15 AM  
(11 8 28)

12-NOON

4 PM

PATIENT N° 3  
1st 24 HOURS  
INTRAMUSCULAR  
N° 693  
DOSE = 300 MG

C1

Percentage of Sb excreted

INJECTION AT  
2-30 PM  
(12 8 28)  
7-15 PM

9-PM

10-40 PM

1-AM  
(13 8 28)

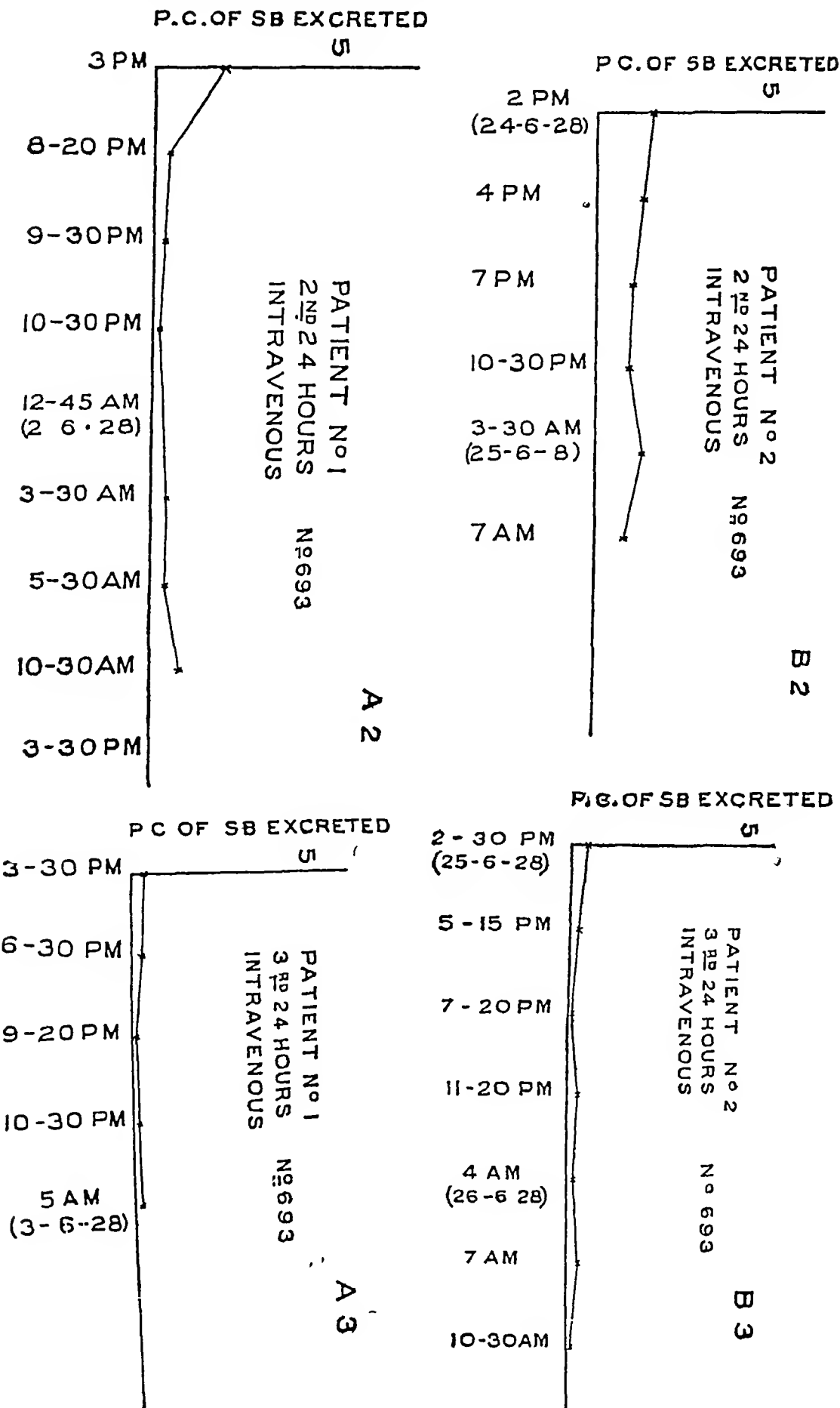
5-30 AM

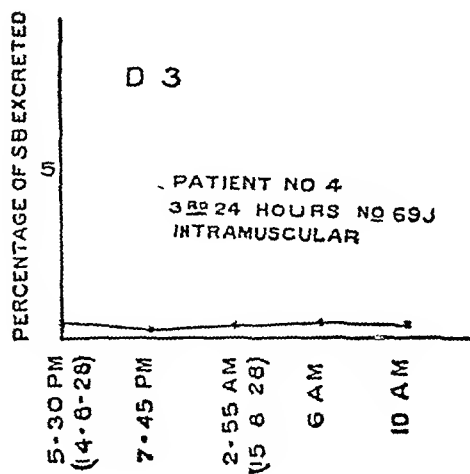
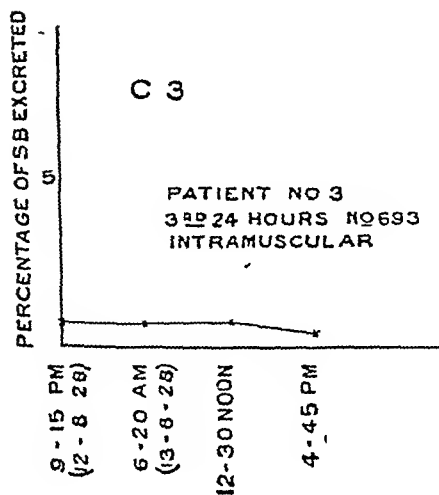
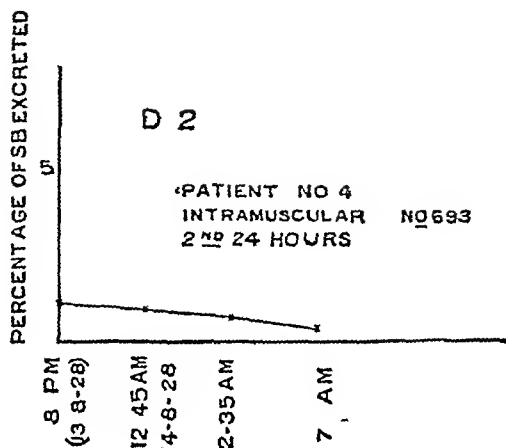
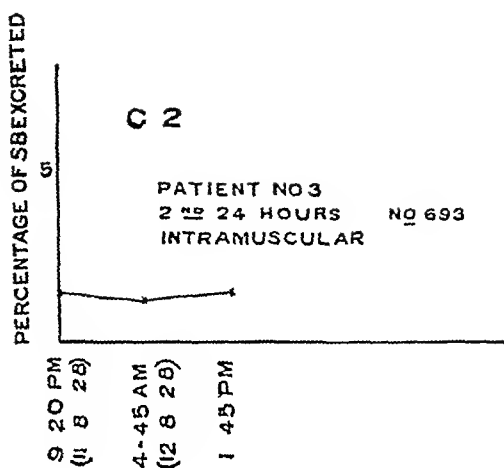
12-45 PM

N° 693  
DOSE = 300 MG

D1

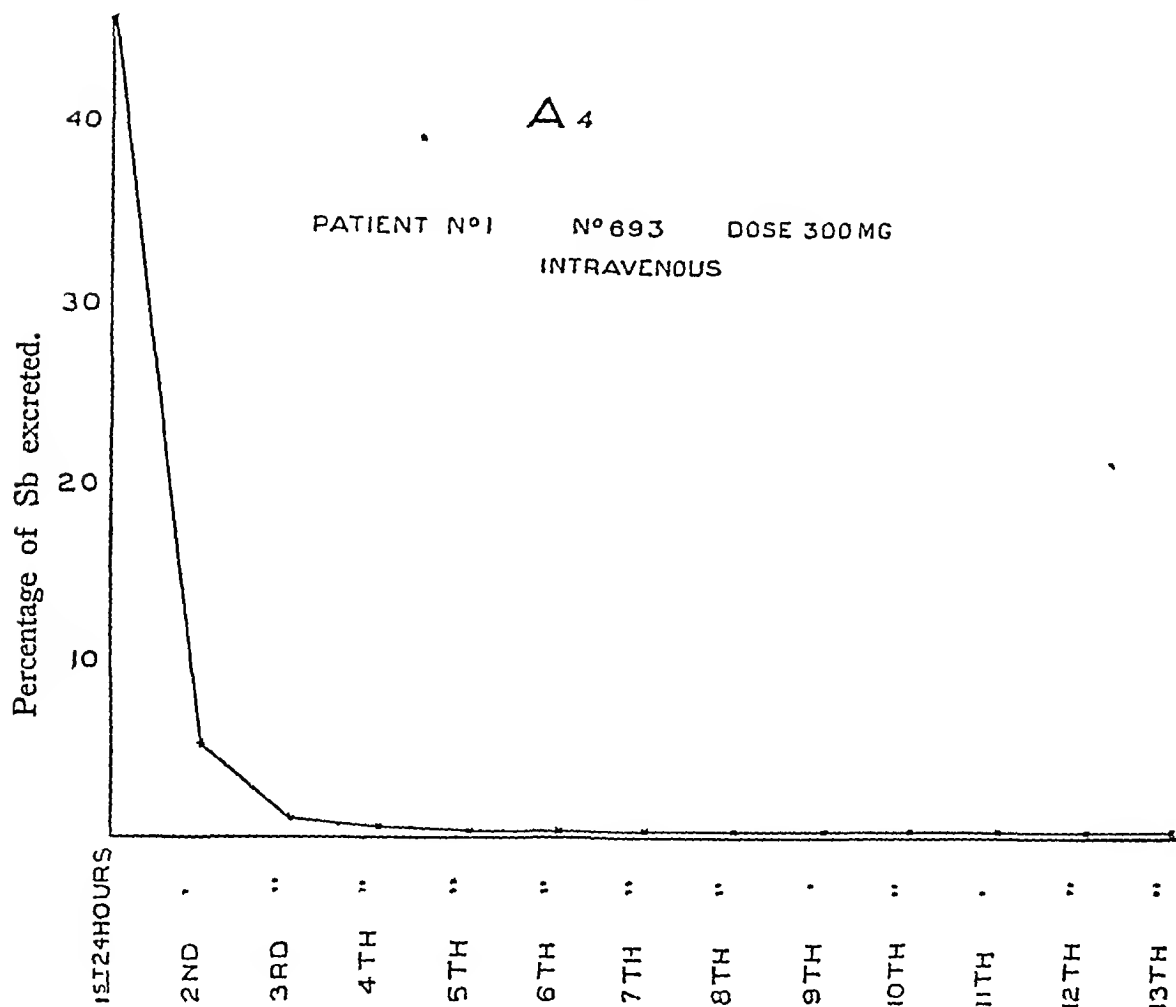
PATIENT N° 4  
1st 24 HOURS  
INTRAMUSCULAR





A comparison of this graph with Graphs A and B shows a close similarity not only in the type of curve, but also in the percentage of antimony excreted on respective days

Our last series of observations were carried out to determine the excretion rate of antimony after administration of antimony in the trivalent form. Two patients were given a single intravenous injection of 50 mg and 60 mg respectively of sodium antimony tartrate and the amount of antimony excreted in the urine estimated in twenty-four hours sample. The results are shown in Graphs



F and G. It will be noted that the graphs are of quite a different type and show a low percentage of excretion during the first twenty-four hours and no such abrupt fall in the excretion rate as with compound No. 693, the graph in fact is characterized by a gradual fall in excretion rate.

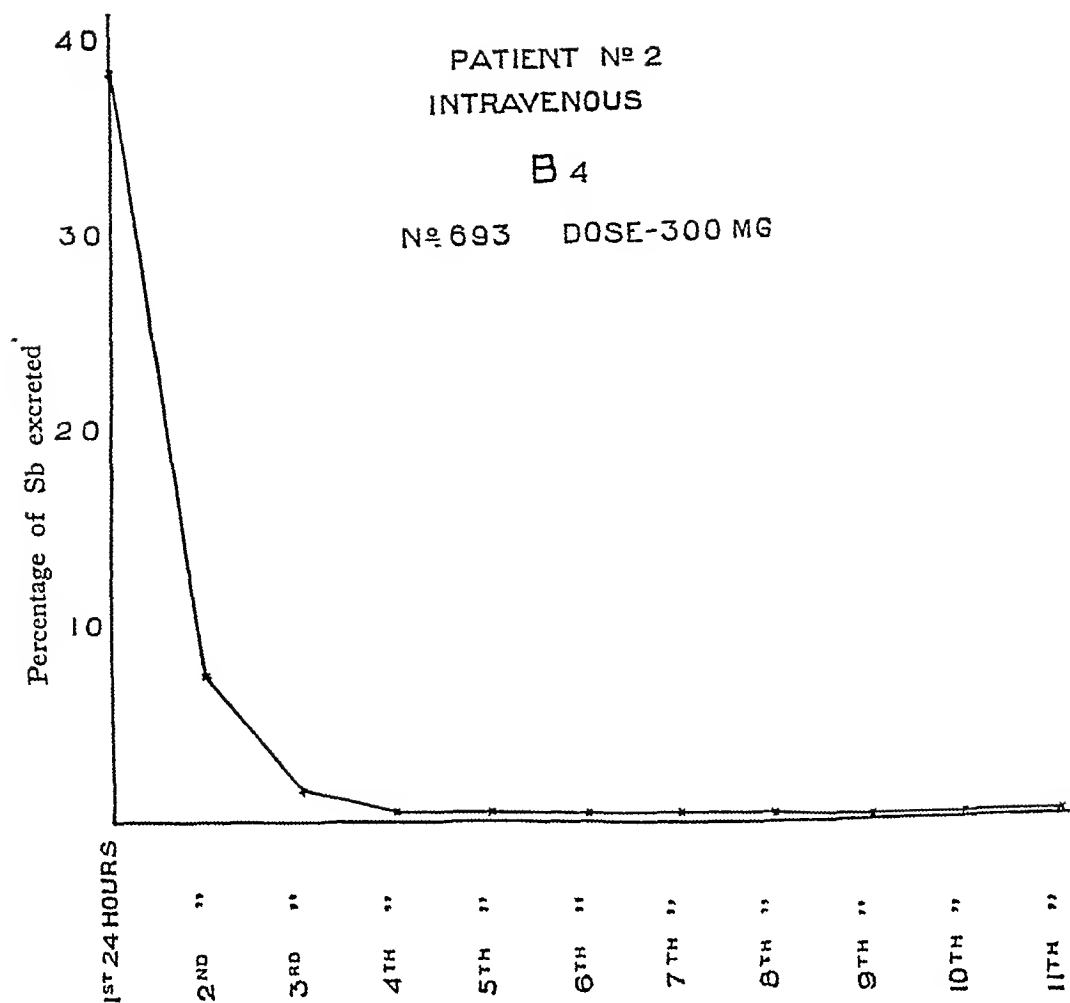
#### SUMMARY AND CONCLUSIONS

(1) We have discussed the question of a suitable method for the estimation of antimony in the presence of organic matter and have given a detailed description of the technique we employed and the probable error.

PATIENT № 2  
INTRAVENOUS

B 4

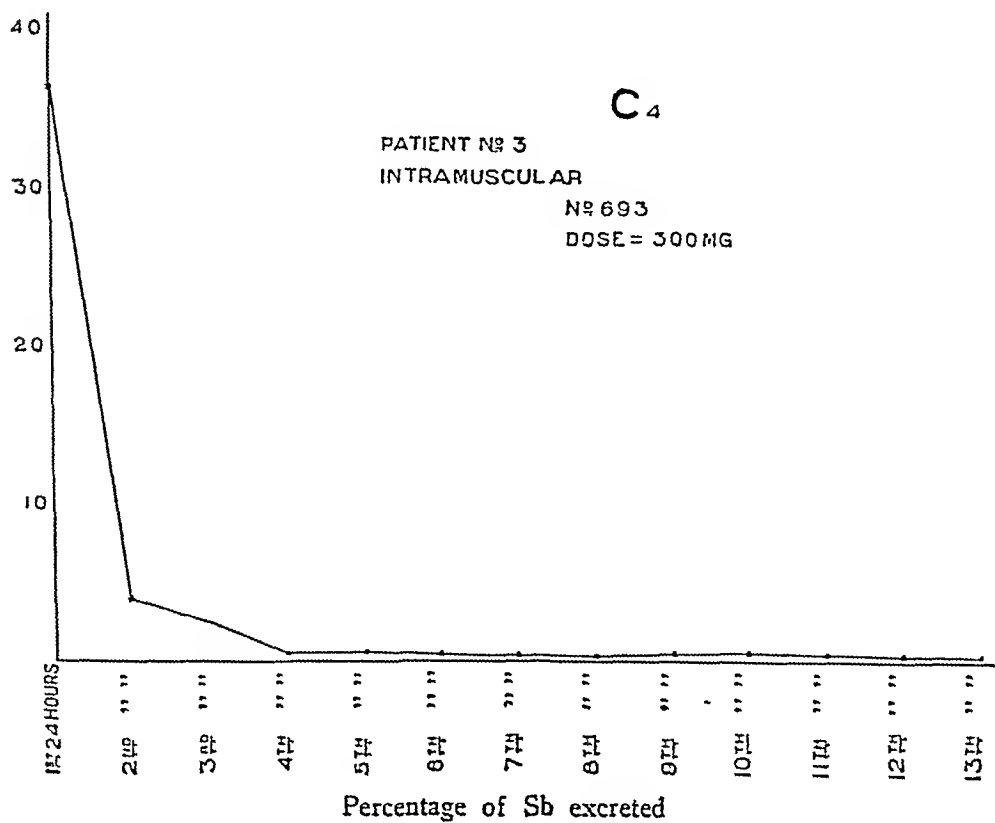
№ 693 DOSE-300 MG

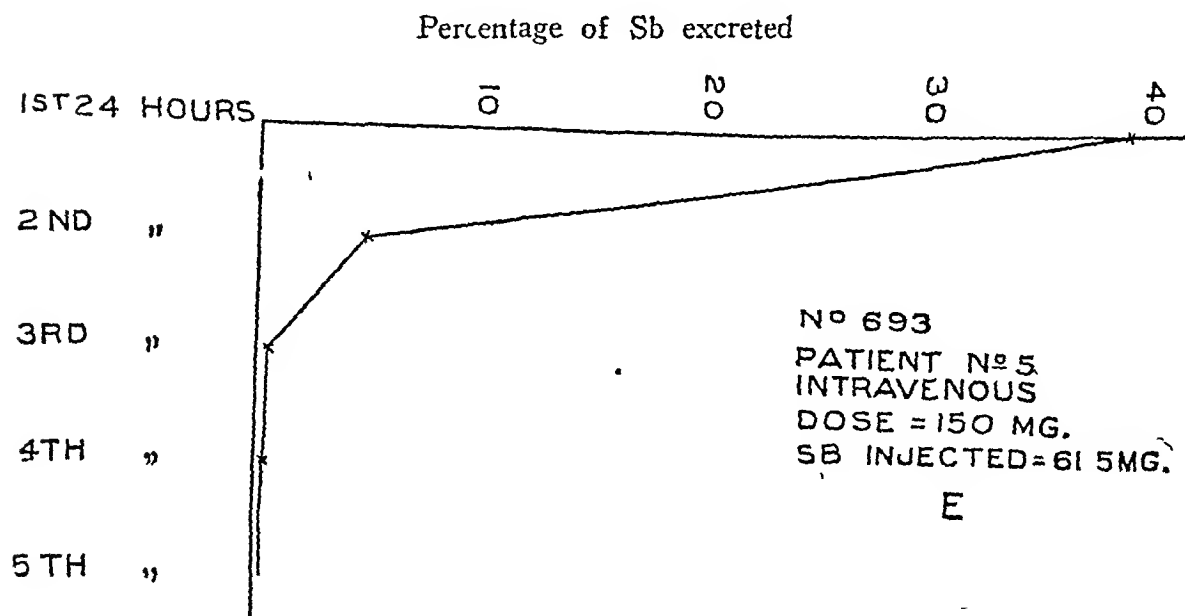
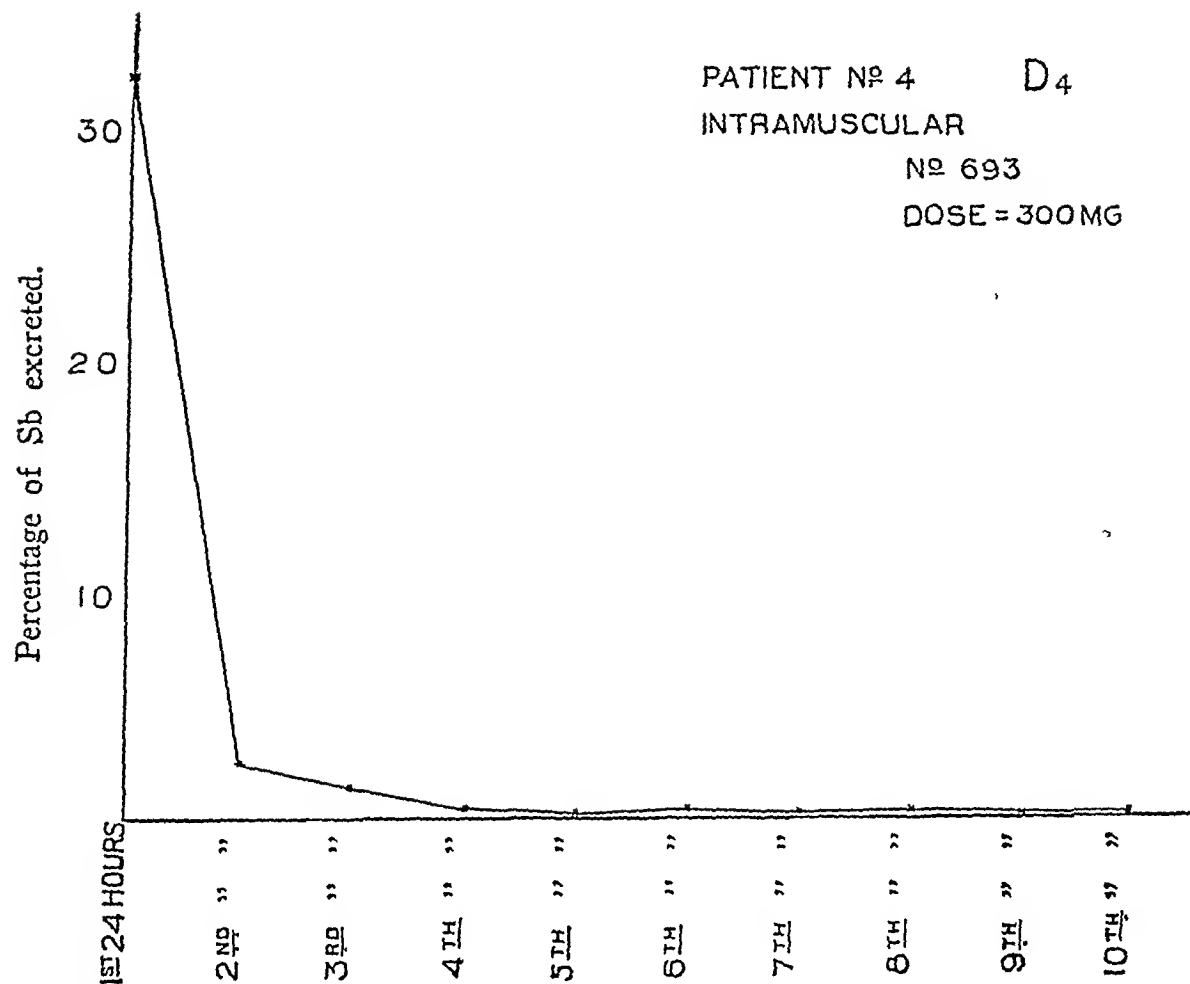


C 4

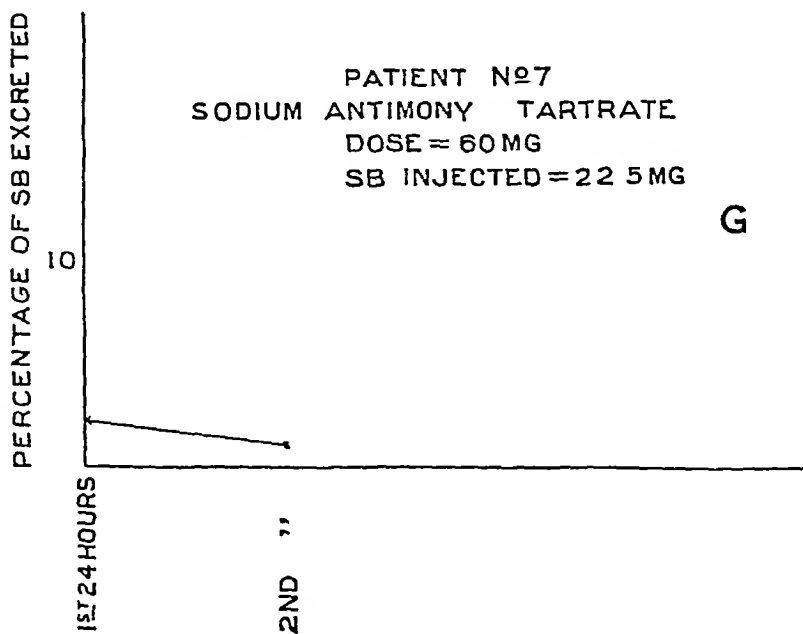
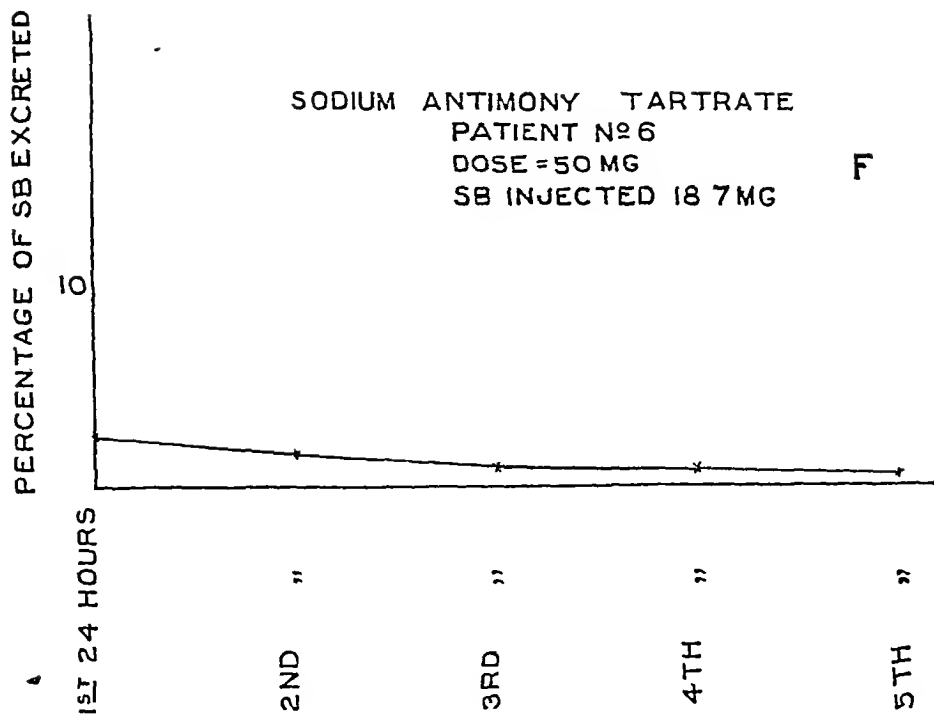
PATIENT № 3  
INTRAMUSCULAR

№ 693  
DOSE = 300 MG



*Observations on Excretion of Antimony in Urine.*





(2) We have studied in detail the excretion rate of a pentavalent compound (i.e., No 693), when given intravenously and found that on an average about 41 per cent of the antimony is excreted in the first twenty-four hours, 6 per cent in the second, and about 1 per cent in the third twenty-four hours

(3) We have shown that when the same compound (No 693) is given intramuscularly about 34 per cent of the antimony is excreted in the first twenty-four hours, 3 per cent in the second, and 1.5 per cent in the third twenty-four hours

(4) The types of curves obtained under both intravenous and intramuscular injections are similar except that there is a slight lag in the excretory rate when the intramuscular route is used

(5) Any alteration of the dose of the compound does not make any difference in the percentage of the antimony excreted in relation to the dose

(6) The excretion rate of a trivalent antimony compound (i.e., sodium antimony tartrate) is extremely low when compared with No 693 and is characterized by a gradual fall

We wish to acknowledge our indebtedness to Dr Napier of the Tropical School, Calcutta, who has very kindly supplied us with the necessary clinical material and for his close co-operation in the work. We have also to acknowledge our thanks to the Indian Research Fund Association for the funds to carry out this work

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# THE NATURE OF THE 'BLACK SPORES' ASSOCIATED WITH THE MALARIA PARASITE IN THE MOSQUITO AND THEIR RELATIONSHIP TO THE TRACHEAL SYSTEM

BY

BRUCE MAYNE,

*Malariaologist, Malaria Survey of India*

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## I INTRODUCTION

IN an examination of the voluminous literature on the parasitology of malarial fevers, one is impressed with the significance attributed to the structures occurring in the insect host known as the 'black spores'. These bodies, first described by Ronald Ross (1898) in connection with the parasites responsible for the production of malaria, were considered only second in importance to the organisms definitely identified with the exogenous cycle of that parasite. During a course extending over a period of fully thirty years they were regarded as possibly of ætiological significance. I have attempted to present evidence obtained from a critical study of these bodies which warrants the consideration of a new view of the nature of the 'black spores'.

These structures have been referred to in the literature as the *black spores* of Ross, the *brown bodies*, or *residual bodies* of Grassi, and the *chutin bodies* or *chutin corpuscles* of Brug and Walch. The structures referred to by the various authors are prominent in appearance, quite easily distinguished with the high dry powers of the microscope when search is made for malaria oocysts. They are commonly banana- or sausage-shaped and often S-shaped or round and usually very conspicuous. They occur generally on the gut wall of the dissected mosquito in clusters of about 0.2 millimetre in size, ranging in colour from yellow to black.

The 'black spores of Ross' have up to the present time been thought to have an association with the oocyst of the malarial infected mosquito and on this account warranted serious consideration in a study of the essential phases of the rôle of the mosquito. An attempt has been made in this paper to assemble all the available references to this question of the 'black spores of Ross,' and

on account of their important historical bearing, to submit rather more complete abstracts than is usual in a short paper

## II REFERENCES TO THE LITERATURE

Ross (1905), writing of the difficulties experienced in settling the question of the rôle of the mosquito in malaria, remarks 'I was temporarily not a little delayed by finding inside the mature pigmented cells certain large brown or black bodies, which I provisionally thought might be connected with their life history. It seemed that these black bodies, occurring as they did actually within the pigmented cells (Plate X fig 10),<sup>1</sup> might be of the nature of sporocysts meant in some way to infect other mosquitoes, so that the infection might not only be carried from man to man by the mosquito, but from mosquito to mosquito, or that they might be meant to infect man, as Manson had thought, through the water'

Ross (1923) gained the impression during his earlier work that a portion of the oocyst along with the sporozoites gave rise to black spores (Plate IX, fig 1), and that in old mosquitoes these bodies get carried away into the tissues possibly producing the disease in the insect. He surmised that the black spores might be intended for a free life in which oocysts and sporozoites were perpetuated from the larval stage of the insect. With this in view, numerous trials were made in infecting birds and larvæ by ingestion. He fed as many as 500 black spores, dissected from mosquitoes, to young larvæ repeatedly without result.

Ross claims justification for his interest in these black bodies inasmuch as they occurred in clusters actually within the ripe oocyst. It was not until 9 months later that Ross found the spores in uninfected mosquitoes and was then convinced that they were only 'parasites within parasites'. Later, he states that the bodies are not an essential stage of the life cycle of the Plasmodia. He offers this explanation 'I was quite right to spend time over the question, as it was not one to be summarily dismissed, and has not been fully elucidated even yet' (1923).

Daniels (1900) suggested a distinctive parasitic function to the black tubular bodies or spores found either in the plasmodia cyst of the infected mosquito's gut or 'all over the body of the mosquito,' originating from ruptured oocysts. He confirmed Ross's earlier views of these black bodies in suggesting that as *resting spores* they might be capable of supplementing the usual cycle in the following manner —

(a) Attaining development outside of the insect host and invading a human host through ingestion or inhalation, and then resuming parasitic habits

(b) Perpetuate the malaria infection through hereditary transmission by contaminating larvæ in water and completing the cycle in the developing imago

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\*To facilitate an understanding of the bodies described by different authors, photographic reproductions of some of their figures are given in the appended Plates (See Plates IX and X)

Daniels states that these black spores are very resistant, undergoing no visible change when treated with potassium hydroxide and survive submergence in water for months

Grassi, (1901) interprets the origin and significance of the 'black spores' of Ross in an exhaustive manner. In all the forms observed, they were always associated with the presence of sporozoites and oocysts. In the latter case they occurred before the rupture of the parasites, and often when sporozoites were still within the oocysts. He interprets the brown spores as immature or atrophied sporozoites or fragments of sporozoites with a brown envelope formed about them (Plate IX, fig 2). He prefers the term 'brown body' to 'black spores' of Ross. He observed these forms in anophelines experimentally infected with malaria by feeding on crescent cases, and more rarely in mosquitoes found infected in nature. A definite classification of brown and yellow brown bodies is given depending on their supposed origin: (a) elongated, arising from sporozoites, transparent in part, presenting several constrictions and with yellowish tinge, (b) short rod, resembling a budding blastomycete divided into fragments from the longer forms, darker in appearance, (c) round bodies, composed of a central colourless layer surrounded by a yellow or dark brown covering, occurring in groups varying from two and three, to forty and fifty. When they are numerous they are present in a rounded mass, either within the oocyst or in a tangled mass with a distinct capsule on the gut wall (Plate IX, fig 3). The latter he terms the 'residual body' which he thinks represents an involution form in the development of the malaria parasites. He disagrees with Ross in the claim that these bodies may be parasitic fungi or are of the nature of the true spores. Grassi assigns several reasons for his contention, citing multiple unsuccessful experiments involving ingestion by human volunteers and attempts to infect larvæ with black spores placed in the water in which they were reared.

Relative to the external conditions in nature favouring the production of the yellow brown bodies, Grassi supposes that the lowering of the temperature may possess an influence. He has observed that in all of the *Anopheles* caught by him in nature, the infestation with these forms occurred only in winter.

Blanchard (1905) prefers to call the 'black spores' 'brown bodies'. He intimates a specific origin associated with oocysts and a derivation from sporozoites. He finds them only in matured oocysts in association with sporozoites and distributed in variable numbers from 20 to 50 in a somewhat granular mass.

Blanchard associates the brown bodies with a regression or involution of certain elements in the specific organism of the infected mosquito. He defines them as degenerative resting bodies and is in agreement with Grassi that the brown forms exist mainly in the hibernating mosquito, being influenced in development by the low temperatures.

Celli (1904) discusses the 'black spores' of Ross and inclines to the belief that they are involution forms of sporozoites. He denies that they are the resisting spores of the English workers or the residua of segmentation of the

Italian authors, which they claimed might be perpetuated through hereditary transmission

Stephens (1905) regards the brown spores as having nothing to do with the malaria parasites in the mosquito, but due to quite an independent infection by some other form of bacterial or protozoan life. He finds them in cysts in which the sporozoites have disappeared, the brown bodies vary much in appearance, so it is evident that the same organism is not always present.

Stephens stresses the importance of confirming the Italian workers' observation that the brown spores have been found only in cases fed on simple tertiary parasites. He quotes Gosio giving data in regard to monthly infections of *A. maculipennis* in Tuscany. The latter author submits information indicating that he regards the presence of brown spores in captured mosquitoes of importance equal to the presence of zygotes and sporozoites. Gosio gives a parallel column of figures of dissected specimens found with brown spores in his table of monthly infections of *A. maculipennis*.

Ruge (1903) observed 'Ross's spores' on the stomach and salivary glands of *Culex pipiens* as S- or Comma-shaped, brownish yellow to blackish brown bodies. They were found to be as long or longer than sporozoites and double in width (Plate IX, fig. 7). Ruge distinguished 'black spores' and sporozoites in the same oocyst and agrees with the earlier suggestion of Ross that the darker bodies are developed from the normal insect sporozoites. He submits additional proof of this relationship by interpreting certain bodies in the cysts appearing as yellow and brown coloured sporozoites as transitional forms between brown sporozoites and 'black spores' (Plate IX, fig. 8). Ruge attempted to demonstrate the parasitic nature of these forms by artificial cultivation and animal inoculation with negative results.

Doflein (1911) refers to the 'Ross spores' which were found only in infected mosquitoes. These he considers to be associated with zygotes and sporozoites in a process of degeneration.

Prowazek (1912) states that Ruge encountered 'black spores' especially in mosquitoes fed on naturally infected sparrows. He mentions that this worker kept these bodies in a solution for three months at room temperature and found them unchanged. When the 'black spores' were maintained at body temperature, they were observed to change in form from the typical S-shape to round bodies.

Prowazek cites the observations of Neumann in which he examined 2,573 specimens of *Stegomyia* mosquitoes and encountered two with 'black spores'. Neumann is quoted as inclined to the view that the 'black spores' represent degenerated sporozoites, and the hypertrophied forms of 'black spores' are distorted sporozoites, which do not exhibit motility.

Thomson and Woodcock (1922) consider that the 'black or brown spores' which invade the oocysts are undoubtedly malaria parasites, which have degenerated instead of normally completing their development. They point out that as they degenerate, these oocytes may be invaded by other parasites of the mosquito, e.g., the *Microsporidia*.

Stephens and Christophers (1908) record the finding of 'black spores' in or about the salivary glands of *A. rossii* (*subpictus*). They describe these spores as brownish-black, curved, sausage-shaped bodies suggesting a mycelial nature.

Similar observations show that some of the earlier workers were very doubtful as to the connections of these bodies. Their descriptions are very suggestive of the chitinization of long pieces of tracheal tubes. Ross, Annett, and Austen (1900) report that although 'black spores' were frequently seen in the African mosquito, *Anopheles costalis*, they were never detected within the capsule of the zygote. They often occurred mixed with what looked like segments of a fungus, within the sheaths of certain muscle fibres, and indeed appeared to have no relation at all to the *Plasmodium*. The evidence relating to the growth of 'black spores' indicated reproduction by fission in the manner of fission fungi. The authors suggest that if the 'black spores' are indeed forms of the malaria parasite, they may enact a role of perpetuating the species through infection of the larva 'though it remains to be proved that they have any connection with the parasites of malaria'.

Castellani and Chalmers (1919) believe the 'black spores' found in the oocysts on the stomach wall of mosquitoes to be protozoan in nature, defining them as hyper-parasites of the malaria organism belonging to the genus *Nosema*.

Flu (1920) discusses the occurrence of 'black spores' in the malaria cyst of human and avian sources giving prominence to Brug's discovery of the presence of chitin in these organisms. He indicates that the invasion of the oocyst by 'black spores' is the result of an over-production of chitin on the part of the insect.

Hindle (1914) states that the frequently expressed suggestion of other channels of infection, besides that of the mosquito, gave a distorted value to the presence of 'black spores' within the parasitic cysts. Black spores, he opines, are a species of *Nosema* attacking the mosquito independently of malarial infection.

Manson (1917) favoured the view of hyper-parasitism suggested by Sambon to explain the function of 'black spores'. He stated that these bodies, which are known to be protozoal organisms belonging to the genus *Nosema*, prey on the malarial oocyst and destroy it just as they prey on the larvæ of *Filaria immitis* encysted within the malpighian tubules of the mosquitoes which subserve their development.

This view has been altered in a later edition of Tropical Diseases by Manson-Bahr (1925). He points out that the 'black spores' and the *Nosema* are distinct, inasmuch as the latter is a microsporidian peculiar to the mosquito, and the 'black spores' are the degenerated cell contents of oocysts which have become chitinized.

Bentley (1910) gives definite parasitic value to the presence of brown spores in his report of a malaria survey of Bombay. He enumerates the number of specimens of *Anopheles stephensi* exhibiting infected stomachs relative to their

association with the presence of brown spores, giving the relative numbers found in the years 1909 and 1910

Ziemann (1924) considers the 'black spores' of Ross have a definite relationship with the malaria parasite and gives prominence to the views of Brug and Walch, emphasizing the chitinization of the contents of the oocysts

Minchin (1912) describes the formation of 'black spores' of Ross as degeneration phenomena found in other protozoan forms in which the cysts degenerate and form masses of pigment. He cites a parallel in Schaudinn's observation of the degeneration in the oocysts of *Cyclospora* and accounts for the similar behaviour in *Amaba* and other protozoa in depression periods due to unfavourable conditions. Here there is a tendency on the part of the organism to deposition of grains of fat or other substances in the protoplasm.

He explains that in the 'depressed' state a great quantity of chromatin is extruded from the nuclei in the form of chromidia which degenerates into pigment. In extreme cases, in the *Actinosphaerium* during a depression period, the protoplasm is bereft of its nuclei and the protozoon changes in colour to a general brownish or darker tint. This is also noted in *Amæba proteus*, where, due to degeneration of chromatin in the nucleus, a mass of brown pigment is formed, which may spread through the whole cytoplasm giving it a brownish tinge.

Minchin states, that in connection with the invasion of the oocyst by black spores, it is commonly observed that these degenerating protozoan organisms are subject to the attacks of parasites.

Hegner and Taliaferro (1924) give 'black spores' and the malaria oocysts equal protozoan rank, referring to the former definitely as parasites. They write 'The oocysts known as 'black spores' were for a long time supposed to be formed as a result of low temperatures, but they apparently are oocysts parasitized by *Microsporidia* of the genus *Nosema*'

Wenyon (1926) relative to 'black spores' writes 'Various suggestions have been made relative to the nature of these "black spores" That they are spores of a microsporidian cannot be entertained, as they do not bear any resemblance to these'. They are supposed by Wenyon to be probably the result of the death and degeneration of the oocyst contents at various stages of its development (Plate X, fig 9). He states that it is possible that chitinous material is deposited in them by the mosquito.

Brug (1916) kept *Culex pipiens* infected with *Plasmodium præcox* for 15 to 25 days finding one-fourth of them infested with 'black spores'. In one instance a mosquito with 130 oocysts on its gut wall exhibited 102 of them with these black structures. Tested by immersion in a solution of 10 per cent of potassium hydroxide at body temperature for periods of 1 to 60 days, he found that they bleached only slightly and were not dissolved. Brug states that Ruge kept these bodies in salt solution for about 6 months and they remained unaltered.

Brug was the first to propose the term 'chitin corpuscles' for the various structures generally known as 'black spores'. He interprets one of his illustrations (Plate X, fig 12) as 'black spores' formed from the wrinkling of the



cyst wall which subsequently becomes chitinized Brug makes the analogy of parasitism with the occurrence of chitinized encapsulated microfilaria in the mosquito He points out that ectodermal cells that are necessary for the formation of chitin are found in the close proximity of the cyst in the form of the tracheæ He says, 'I have not ascertained the intimate relationship of the tracheæ to the oocyst'

Brug makes the unusual suggestion that the oocyst ruptures as the result of this chitinization of its contents, a process which he considers of a similar nature to the rupturing of the schizonts of the malaria parasites in the body of man during a paroxysm in a case of tertian or quartan fever

The process of chitinization is said by Brug to occur at the following stages of development —

- 1 Previous to the formation of sporozoites
- 2 When the oocyst is full of sporozoites
- 3 At a time when there are only a few sporozoites in the cyst
- 4 When the so-called 'rest bodies' have formed, and later when the rest bodies have disintegrated

He regards the strain of *Plasmodium* with which he worked as exceptional on account of the following —

- 1 Its great pathogenicity for the canary birds, nearly all of which died at the height of the infection

- 2 The extraordinary frequency of the formation of black spores in the parasitised mosquitoes He found fully one-fourth of the dissected mosquitoes possessing the 'chitin corpuscles'

Brug considers it possible that there is here a specific correlation, the heightening of the virulence of the strain of bird malaria due to the presence of the chitin corpuscles in the mosquito and, vice versa, the increase in numbers of these chitin corpuscles in the mosquito in proportion to the severity of the malaria infection in the bird

Walch (1922) explains that the name 'black spores,' except for its historical significance should be abandoned, the distinction of colour and relationship being only arbitrary He supports Brug in proposing to change the name of these bodies to 'chitin corpuscles' He confirms Grassi and Brug in finding these chitin bodies of colours varying from yellowish to black, yellow brown, and decidedly brown and black He found also that they were not constant in form and size, varying from spherical and S-shaped to banana-shaped, the last seeming to predominate (Plate IX, figs 5 and 6)

Many of these chitin bodies were observed by Walch in non-infected as well as malaria infected anophelines, finding them in the thorax as well as the gut and ovaries, and he agrees with Brug that the brown bodies of the mosquito represent parts of the oocysts transformed into chitin He thought the chitinization was comparable to the calcification taking place in human pathology and regards Fulleborn's observation in finding a filarial larva surrounded by a

association with the presence of brown spores, giving the relative numbers found in the years 1909 and 1910

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Brug was the first to propose the term 'chitin corpuscles' for the various structures generally known as 'black spores'. He interprets one of his illustrations (Plate X, fig 12) as 'black spores' formed from the wrinkling of the

were found to remain unchanged in some of these solutions for a maximum of ten months. Several specimens resisted the action of caustic potash for as long as ten days.

### *A Morphology and Classification*

I have measured probably several thousands of the structures finding them quite uniform. In thickness they are from 0.7 microns to 3.0 microns (the majority measure 2.5 microns), and the lengths, roughly the same as many sporozoites of both human and avian types of malaria, are within the limits of 7.0 microns and 16.0 microns. In one instance the length was fully 20 microns but this is exceptional.

The round bodies, the so-called 'resting' or 'resistant' bodies, vary considerably more, the dimensions of these I have found to be about 5 microns to 20 microns.

The Ross 'black spores' have been seen in individual forms, in broken lines end to end, in small clumps of distinct curved bodies, and in matted masses of hundreds resembling mycelial threads of a fungus.

These various forms may be conveniently classified into the following types —

#### *Type 1*—Banana, curved or sausage-shaped type

This is the prevailing form, occurring principally in clumps of 15 to 20 (*vide* Plate XI, fig. 13), and less frequently in isolated examples scattered through muscular or connective tissue of the thorax and intestinal tract. A rare form is the S-shaped structure occurring singly or combined with the usual group of banana-shaped 'black spores'. This variety appears as the extended banana form, apparently adapting itself to a bend of the tissue. It is seen along the curved edge of the midgut wall (*vide* Plate XII, fig. 18).

#### *Type 2*—The round or disc-form type

These are usually of larger diameter than the oval or curved forms. They are described as generally having a clear centre and a dark periphery. They appear seldom in isolated form, but occur more commonly in a group of other structures giving the appearance upon close inspection of the longer forms in optical section.

#### *Type 3*—The mass formation type

These bodies, mainly of Type 1, may occur in clumps, often in such numbers as to form large oval masses, penetrating the muscular tissue of the gut wall to an appreciable depth. In that state the more deeply situated bodies are often obscured and only the top superficial layer can be clearly distinguished (*vide* Plate XI, fig. 14). The former are, however, of similar shape to those occurring on the surface, either in groups or singly. These masses, of as many as 100 or more individual forms, often occur in great numbers sometimes to the extent of 30 to 50 in one specimen.

#### *Type 4*—The linear type

This is the form resembling strikingly the mycelial branches of a fungus. Its shape is generally a long oval, occurring often in disconnected lines distributed

through the muscular tissue. It is seen more commonly in the thorax. These 'black spores' may appear in single lines broken by interspersing tissue (*vide* Plate XI, fig 15) or forked lines either on the gut wall or in the thoracic muscles.

*B Regions of the Mosquito where 'Black Spores' were observed*

The writer has observed in the ample material studied all the forms of 'black spores' previously defined by him and described by the various workers in all the situations ascribed to them. He concurs fully with the descriptions of form and structural details of the original 'black spores' of Ross and Daniels, appreciating in the fullest the logical modifications of the nomenclature of 'brown bodies' suggested by Grassi and 'chitin corpuscles' by Brug and Walch. I have found these forms in the insects' bodies in similar locations to those mentioned in the recorded observations. They include the following —

- I In or on the oocyst, singly and in masses, both before and after sporozoites were formed
- II On the gut wall independent of oocysts, on the hind-gut more rarely than the midgut
- III On the ovaries, in various stages of development except on the fully formed egg
- IV Within muscle tissue of the torn dissected thorax.
- V In or near the salivary glands

The following data are given to show the distribution in the insect's body of these chitin depositions. Here account is taken only of the appearance of these bodies in one or more clumps, single isolated forms are not included.

TABLE I

*The number of mosquitoes with 'black spores' and their location in the insect*

Position	Number of specimens observed
On or in oocysts	8
Gut wall	25
Thorax tissue	8
Salivary glands	6
Glands and muscle in proximity	4
Ovaries	8
Lines of black forms along trachea	7
TOTAL	66

Some of the apparently unusual places one finds them are on the wall of the œsophagus (rarely), the hind-gut (more often), and on the salivary glands (quite commonly). Mention has already been made of their occurrence on the ovaries and in the muscle fibres of the thorax. I have never encountered the bodies on the malpighian tubules, although it may be interesting in this connection to note that on two occasions sporozoties were observed moving actively within the cells of the malpighian tubules of infected *Culex fatigans*.

It may be pointed out that the occurrence of these chitimized bodies on the ovaries also argues against any relationship to the malaria parasite, because sporozoites have never been found within the ovaries

Another interesting point should be mentioned, I have found that although 'black spores' vary in size relative to each other, they do not adapt themselves to the size or stage of development of the invaded oocyst. Small oocysts appear to have 'black spores' of the same size as developed ones, young oocysts, prior to the 5th day of incubation, have 'black spores' of the same size and kind as those on older oocysts with sporozoites

### *C Influence of Malaria Parasites on the Presence of 'Black Spores'*

An effort was made in a parallel, controlled experiment to determine what influence the presence of malaria parasites exerts on the production of the bodies known as 'black spores'

A sparrow, whose blood showed on repeated examinations the absence of plasmodia, was placed in a cage with about 180 specimens of laboratory-bred *Culex fatigans*. The following morning 126 engorged mosquitoes were removed, separated into lots of five or six in netted globes, and placed in the most favourable conditions available in the laboratory

Two hundred of the same strain of laboratory-bred *C fatigans* were allowed to feed the following evening upon two sparrows which had shown a heavy infection with *Plasmodium præcox*. One hundred and thirty engorged mosquitoes were removed from the feeding-cage the next morning and subjected to the same conditions as the previous group. After eight to twelve days the two lots were dissected and the results tabulated as follows —

TABLE II

*Showing results of feeding C fatigans on infected and healthy sparrows*

Date	Number of days after blood meal	Number of mosquitoes dissected	Number of uninfected mosquitoes with 'black spores'	Number with parasites and 'black spores'	Number with parasites without 'black spores'
<i>A Fed on healthy sparrows</i>					
August, 11	8	46	4		
" 13	10	25	3		
" 14	11	25	10		
" 15	12	30	6		
<i>B Fed on infected sparrows</i>					
August, 12	8	29	1	2	21
" 13	9	35	3	3	25
" 14	10	30	1	3	18
" 16	12	36	0	4	28
Percentage of infected mosquitoes with 'black spores'					10.1
Percentage fed on healthy bird with 'black spores'					10.8

## SUMMARY OF TABLE II

Of 126 specimens of bred *Culex fatigans* fed on a sparrow, presumably free from malaria infection, 23 were found with 'black spores' after 8 to 12 days

Of 130 specimens of bred *Culex fatigans* fed on two sparrows, whose blood showed a moderate number of gametocytes, 104 after an incubation of 8 to 12 days exhibited numerous oocysts and sporozoites. In this set 5 of the mosquitoes which proved free from infection developed 'black spores,' in addition to 12 specimens which harboured malaria parasites

In this single observation the presence of plasmodia in the mosquitoes appeared to have no specific influence on the production of 'black spores'. In both trials, involving infected and uninfected mosquitoes, about 10 per cent showed the chitin bodies under parallel conditions

Some evidence against any relationship of those bodies to malaria parasites is offered in the following data which may be applied as a control in the experiments involving *Culex fatigans* and avian plasmodia

In an effort to determine the possibility of an anopheline mosquito acting as a host of bird malaria, experiments with *Anopheles subpictus* resulted in the finding of eight specimens infected. Both artificial and natural cases of harbouring of plasmodia were observed. Oocysts and sporozoites were found in the two series. A preliminary account of these observations is reported elsewhere (Bruce Mayne, 1928)

In these experiments 164 specimens of *A. subpictus* and *A. culicifacies* were dissected and examined. In regard to the presence of malaria parasites and 'black spores,' the following were the findings recorded —

Number of anophelines found to harbour bird malaria organisms	8
Number of these found with 'black spores'	0
Number of mosquitoes free of malaria organisms	156
Number of specimens found with 'black spores'	4

*D Notes on the Occurrence of 'Black Spores' in the Absence of Malaria Infection*

A large amount of material was examined which was selected on account of its freedom from any possible contact with malaria infection. It comprised dissections of the following insects —

1 Males of bred and wild *Culex fatigans*, *Anopheles culicifacies* and *A. subpictus*

2 Females of *Anopheles culicifacies* and *A. subpictus*, bred specimens, which were not given an opportunity to obtain blood

3 Non-blood-sucking forms, such as house flies and other muscids

A record is submitted of the extent and form of the bodies identified as 'black spores' as observed in the insects examined. Doubtful forms are excluded in this category

*I Number of male mosquitoes showing 'black spores'*

- (a) *C fatigans* one of 20 bred specimens examined

One 'round' form (Type 2) upon the hind-gut involving the wall of a tracheal tube and plainly visible as part of the tube. It appeared heavily chitinized, dark brown in colour in one of the larger tubes which at this point measured 11.5 microns in thickness. The round mass of chitin measured 10 microns.

- (b) *A subpictus* two of 34 wild specimens examined

(i) On the anal segment an oval shaped chitinized body apparently continuous with a tracheal tube.

(ii) Another specimen with a spherical mass (Type 3) measuring 45 microns composed of many bodies forming a black aggregation on the wall of the oesophagus (about midway). It resembled strikingly the 'residual bodies' of Grassi.

- (c) *A subpictus* one of 22 bred specimens examined

Dissected a few hours after emerging. Two small clumps of brown 'black spores' of 2 and 3 bodies (Type 1). Also 2 masses of 9 each. In addition a few scattered single light brown forms were noted.

- (d) *A culicifacies* three of 48 bred specimens examined

(i) Two examples of single forms on the gut wall. One, lying in the tissue of the mid-gut wall, a typical dark yellow curved form, measuring 7 microns long and  $1\frac{1}{2}$  microns in thickness. Another, a brown form apparently broken from a tracheal tube on the gut wall. (Both Type 1).

(ii) Two individual dark brown spores (Type 4) along the tracheal line in the muscle tissue of the thorax near the salivary glands. They measured about 11.0 microns.

(iii) A dumb-bell-shaped mass (Type 3) within the tissue in the anal region. This was quite black and gave the appearance of the exposed ends of several small chitinized tracheal tubes. In addition, on the mid-gut, a line of brown forms (Type 4) along a trachea resembling the chitinized structures referred to as 'hyphomycete' by Walch (Plate IX, fig. 4).

*II Number of female mosquitoes showing 'black spores'*

*Anopheles culicifacies* four of 25 bred (freshly emerged) specimens

(i) Upon the ovaries, 2 brown structures (Type 1) connected with a tracheal tube, appeared as the usual chitinized 'black spores'.

On the malpighian tubules, one instance of round variety (Type 2) of 'black spores,' the so-called 'resting body' of Grassi.

(ii) A mass of about 18 chitinized S-forms (Type 1) alongside of the spermatheca. Two instances also of single spores along the tracheæ on the gut-wall.

(iii) Overlying a malpighian tubule, one instance of a brown body seen under 1/12 inch oil immersion objective seemed to be wholly within the tracheal tube, length about 8 microns

In the thorax one tracheal tube piercing the muscle tissue containing four short lengths, 5 to 10 microns (Type 4), of chitinized light brown forms

(iv) A group of 3 forms (Type 4) on the hind-gut, 8 to 10 microns long, of brown colour with obviously lighter centres, connected with a tracheal tube. In addition 2 groups of 10 to 18 dark brown forms (Type 1), and 3 isolated bodies, within the connective tissue of the anal segment beyond the rectal glands

### *III Number of muscid flies found with 'black spores'*

House flies four of 43 specimens examined

(i) Two females In one, a line of 3 black spores (Type 4) connected with tracheæ on the gut-wall. In the other, several forms indistinguishable from 'black spores' (Type 1) over the ovaries

(ii) Two males In the first, a mass of chitinized bodies connected with a large tracheal tube, seven in number (Type 1). They appear to be made up of smaller tracheal structures with chitin deposits. The other, with two bodies suggesting 'black spores,' one on the rectal papillæ, the second connected with a tracheal tube of the rectum

## **E THE INFLUENCE OF EXTERNAL CONDITIONS**

### *(1) Humidity*

In attempting to determine the effect of external conditions on the appearance of 'black spores' in the insect, a large number of observations on the influence of low relative humidity were available and applicable to this study

Specimens of *Culex fatigans* were subjected to a wide range of relative humidity from 40 to 100 per cent during the hot season of 1928 when temperatures up to 96°F with a mean daily maximum of 86°F were recorded in the insectary. In this connection bred specimens, used in testing the effect of various degrees of humidity on the life of the mosquito and the viability of the malaria parasites it harboured, were studied. This material may be assessed as to the concomitant relation of humidity and the production of 'black spores'. In the series under discussion 274 mosquitoes are considered, a total of 221 of these harboured oocysts of avian plasmodia, and in these infected specimens there were observed 55 instances of 'black spores'. In the 53 uninfected specimens there came to notice 11 examples of 'black spores,' the proportion being about equal to those in which oocysts were found



In the following table records are given of the association of humidity with the presence or absence of 'black spores' —

TABLE III

Relative humidity recorded	Total number of mosquitoes exposed	Number with 'black spores' present	Number with 'black spores' absent
40—45 per cent	27	6	21
40—50 „ „	53	13	40
50—63 „ „	61	15	46
70—85 „ „	91	22	69
85—100 „ „	42	10	32

SUMMARY OF TABLE III

Variations in relative humidity appeared to have no influence on the production of 'black spores' in mosquitoes. Approximately, 22 to 24 per cent of mosquitoes produced 'black spores' in each of the conditions of humidity to which they were exposed.

The specimens of *Culex fatigans* subjected to low relative humidity during the hot season were found to have a greater number of 'black spores' in masses than those kept at high relative humidity. This was observable particularly in specimens which after being kept at a low humidity were not dissected until the mosquito was in an enfeebled state or immediately following its death.

### (2) The Influence of Low Temperature

An opportunity was presented of testing the theory, discussed so frequently in the literature, that 'black spores' may be due to the 'involution' or 'regression' of oocysts under certain temperature conditions.

The material available was as follows —

A large number of bred *Culex fatigans* were fed on sparrows infected with *Plasmodium praecox* during the period October 20th to 26th.

These mosquitoes were kept under similar conditions to the lot studied during the hot season. Up to the time of the completion of these observations, January 30th, 1929, mosquitoes were dissected at intervals until a total of 90 had been examined. Of this number 67 proved to harbour parasites, the majority, oocysts and sporozoites at the same time.

A critical examination of this material showed that the black spores, so abundant previously, were almost entirely absent.

At this time experiments on the effects of lowered humidity were tried with non-infected anophelines, the control material of which was kept under conditions similar to that of the specimens of *Culex fatigans*. These consisted of 112

specimens exposed at temperatures of about 40° to 60°F, and relative humidity, under artificial conditions, varying between 38 per cent and 65 per cent. The control conditions provided for the same temperature and humidities of 65 to 92 per cent. The species represented were *Anopheles culicifacies*, *A. subpictus* and *A. fuliginosus*. It was interesting to note that only a single specimen of these exhibited bodies resembling black spores (Type 1). It should be mentioned that during the hot season, these forms had been observed in several specimens of the species of anophelines used in these experiments.

Relative to the infected *C. fatigans* of this series, a special point was made of examining carefully the contents of every oocyst encountered in the infected mosquitoes in order that one might detect any evidence of 'black spores' of such a form that they could be interpreted as due to steps in a process of regression or involution of the oocyst. Such forms were not observed either in the hundreds of developing oocysts or in the discharged cysts represented by their capsules. There would have been ample time for development of such forms in these mosquitoes which were kept under observation up to 90 days of infection through the cold season.

Only twelve specimens of this entire series exhibited bodies resembling 'black spores'. The forms present were individual round bodies (Type 2) of a light brown colour scattered on the gut-wall to the maximum number of eight in any specimen. In addition eight mosquitoes were observed with a few isolated S- or oval-shaped forms (Type 1) connected with tracheal tubes on the gut-wall. No example of a mass of 'black spores' was observed.

### (3) *Effect of Injury*

It was suggested by Major J. A. Sinton that the formation of 'black spores' in the mosquito might be influenced by some mechanical or traumatic injury. This idea gained credence in observing a specimen of *Anopheles subpictus* emerging from a pupa kept in a breeding-jar. This specimen appeared damaged directly after its emergence. It was dissected the following day, when on its gut-wall were seen several single banana-formed 'black spores'.

The effect of external injury was tried with 38 specimens of wild and bred *Culex fatigans* by shaking violently the glass jars holding the experimental specimens for a few minutes each day for a period of two weeks. Although the mosquitoes did not live as long as other specimens not disturbed by jarring, they gave no evidence of greater susceptibility to the production of 'black spores'.

The suggestion that the blackening and hardening of the tracheal tubes in the process of chitin deposition may be due to mechanical obstruction, injury or other external agency is confirmed in a parallel instance among the honey-bees of temperate climes.

Bullamore (1922) mentions the discovery of a mite in the tracheæ of honey-bees causing a pathological condition of which the blackening and hardening of the tracheal tubes was a marked accompaniment.

Fulleborn, quoted by Brug (1916), has suggested that the chitinization leading to deposition of 'black spores' is quite analogous to the presence of chitinized or calcified filarial larvæ in the thorax of the mosquito host (This analogy is pertinent only if one accepts the view that 'black spores' are parasites) I prefer to accept the analogy to the calcification, particularly that of the respiratory tract, taking place in human pathology mentioned in a general way by Walch (1922)

#### IV THE TRACHEAL ORIGIN OF 'BLACK SPORES'

As the work recorded in this paper points to the chitinization represented in the 'black spores' being definitely associated with the tracheæ, it is necessary to give a brief summary of the histology of the latter structures

##### A Anatomy and Histology of the Tracheal System

Sharp (1910) writes 'The structure of the tracheæ is remarkable. They are elastic and consist of an outer cellular and an inner chitinous layer, this latter is strengthened by a peculiar spiral fibre which gives the tubes a transversely closely striated appearance. The spiral fibre is absent in the fine capillary twigs of the tracheal system. The mode of termination of the capillary branches is not clear. It is supposed that they terminate by penetrating cells or that they simply come to an end with either open or closed extremities

All the organs are abundantly supplied with a capillary tracheal network or arborescent ramification and in some cases the tubes enter the substance of tissues near their termination'

Packard (1909) states, that the distribution of the air tubes depends upon the shape of the organs, varying in size accordingly. The tubes ramify in all directions around the large hollow organs such as the digestive canal, forking so that the branches diverge at a wide angle. Around the organs of more elongated form, the tracheæ run more longitudinally, as is shown by the air tubes of the muscles. Here a short thick trunk arrives at the muscular bundle and, dividing very rapidly, breaks up into a large number of delicate tubes, which penetrate between the muscular fibres, terminating in tubes of exceeding fineness forming what appears like an interlacing network

Miall and Denny (1866) refer to the tracheal system of insects in general, stating that the first stages of development is noted in the tracheal ramifications, which are not formed by a process of direct invagination but by the separation of chitinogenous cells, which cohere into strings followed by irregular tubules. The cells are found to secrete a chitinous lining, afterwards losing their distinct contour fusing to a continuous tissue. At the time of moulting, the chitinous lining of the tracheal tubes is cast. The chemical stability of chitin is 'so remarkable that we might well expect it to accumulate like the inorganic constituents of animal skeleton and form permanent deposits'. It changes slowly under the action of water, keeping for a year in this medium. The colouring

matter of the chitin is amber-yellow in thin sheets and blackish-brown in dense masses

Folsom (1923) writing of the structure of the air tubes states, 'the chitinous lining or intima is thickened at regular intervals to form thread-like ridges which course around the inner circumference in essentially a spiral manner, though the continuity of the spiral thread is frequently interrupted'

Lowne (1895) gives the measurement of the tubes of the largest tracheal trunks as  $1/400$ th of an inch in diameter (60 to 80 microns) In the smaller tracheæ, the tubes end in capillary vessels from 2 to 3 microns wide

Patton and Cragg (1913) write relative to the tracheæ of the mosquito, that they represent invaginations of the ectoderm, and as such are lined from the spiracle to the final divisions with chitin, which is continuous with the exoskeleton The surface, which is in contact with the body contents, is lined by a layer of cells which is continuous with the hypodermis or chitinogenous layer of the exoskeleton The internal lamina consists of a very thin but an uninterrupted layer of chitin, but in all except the most minute tracheæ this is modified to form the well known spiral thread The intervals between the turns of the thread are filled in by a very thin layer of chitin

The outer or hypodermal lamina of the tracheal wall, which is in contact with the internal organs, is very thin, 'continuing to form a sort of peritoneum within which the internal organs are contained'

#### B The Connection of 'Black Spores' with the Tracheæ

Strong evidence of the tracheal origin of the chitin bodies was noted in many mosquitoes, in both infected and uninfected specimens of at least three different species In many individuals the tracheæ appeared as prominent tangled masses, in isolated groups of dark or light brown masses of rather definite shapes and sizes in muscular and epithelial tissue

In the specimens studied in India one sees occasionally, within the oocyst particularly, disc-like bodies in the place of the banana-shaped forms of the usual type of 'black spores' These are thickened hollow ring-like bodies of the same chitinous structure as the others Several observers have noted and described these forms, laying stress on a connection, more or less obscured, with the development of the sporogonic cycle of the malaria parasite

I consider it logical to interpret these chitinous rings as broken-off portions of these branched tracheæ remaining on the site where there occurs a capsule of a ruptured oocyst When present in uninfected mosquitoes, they appear like cross sections of chitinized deposits in the tracheal tubes More rarely they occur as double disc-like forms, and in several instances I have seen revealed the spiral tracheal connections Groups of four to six of these dark rings were observed on the gut-wall and single ones especially during the season of low temperatures

One may fairly make a general statement that the 'black spores' are found usually associated with the tracheal system regardless of the presence or absence

of oocysts and sporozoites. They have been observed to be predominant where the tracheal tubes are most numerous.

Specific instances of tracheal association, selected from a large number observed, are given in detail in the following notes —

#### IN '*ANOPHELES SUBPICIUS*'

*Specimen No 241*—A female specimen showed several masses of 'black spores' which were observed under the 1/12 inch oil immersion lens and high power ocular. One of these 'spores' had a torn end which revealed the tubular interior as well as the spiral envelope.

*Specimen No 89*—There were discerned on the gut-wall 3 masses of 'black spores' of the usual S- and banana-forms. One of these clumps covered an area of 175 microns by 60 microns, bound up in a jumble of tracheæ like a nest or the cocoon of a flea. Among these tracheæ, when viewed by critical illumination, were seen chitinous thickenings constituting typical 'black spores'.

*Specimen No 246*—A female *subpicius* caught in nature was kept for two days while it was fed on the juice of raisins and water. The gut, when dissected, showed tracheæ along the edge of the gut-wall which gave the appearance illustrated in the camera lucida drawing in Plate XII, fig 18. There were two 'black spores' (Type 1) continuous with a tracheal tube. One of these was much smaller than the usual sort encountered, and appeared in a part of the trachea, where the tænidia could not be distinguished, a short distance from a branching tracheole. The other chitin-body was quite typical in structure and shape, and was wider than the trachea in which it originated. The trachea at this site measured 2.5 microns in thickness and was completely replaced by the black S-shaped chitin-body which was about 20 microns in length. This specimen was examined in a fresh state by several of my colleagues who admitted fully the tracheal relationships as shown in the figure.

*Specimen No 252*—Male *subpicius* newly emerged in laboratory. Embedded in the wall of the anterior part of the mid-gut could be easily discerned two masses of 'black spores' (Type 1), one quite black, within the connective tissue, the other on the surface not embedded in the tissue. The latter appeared of smaller size. Three of the dark bodies of the first-mentioned groups were somewhat isolated and their spiral envelopes could be made out.

#### IN '*CULEX FATIGANS*'

*Specimen No 255*—A mosquito in advanced stage of infection, harbouring sporozoites. On the gut-wall there were 5 oocyst capsules. One well-formed capsule contained 7 'black spores' (Type 4) in two lines along the edge of the oocyst. In these bodies the hollow interior and spiral markings of the transformed trachea could be made out.

*Specimen No 29*—This is one of several specimens in which the 'black spores' are found in straight or broken lines in the thoracic muscles (Type 4). Here there appeared one straight line of these bodies and several shorter ones suggesting segments of tracheæ embedded in the tissue (see Plate XI, fig 15).

Four of the dark forms, when viewed with 1/12 inch oil immersion lens, showed quite plainly their tubular interior and vestiges of the transparent spiral tænidia apparently surrounding the black body.

*Specimen No 93*—Here there was a discharged oocyst with a mass of black spores composed of 15 to 20 pieces (Type 3). They were quite jet black though the tubular structure could be readily discerned when viewed with a bright light.

*Specimen No 76*—On this specimen there was seen embedded in the muscular tissue of the thorax two lines of single black spores numbering 22. They were quite typical in form and colour. They measured 12 to 15 microns in length and 2 to 2.5 microns in thickness.

In addition a single clump of 9 spores (Type 4) was seen situated in the muscle fibres close to a large trachea (similar to that in Plate XI, fig 16).

*Specimen No 194*—This was a heavily infected specimen with numerous oocysts. A few of these contained small clumps of chitinated bodies (Type 1) either on the capsule or apparently directly beneath the parasite covering. On the muscular gut-wall the tracheal system gave a decided impression of concentration in the region where there were observed 12 quite typical black spores (Type 1). On close inspection, intimate connection with the tracheal tubes was suggested in the instance of all 12 bodies. The mass of tangled tracheæ (Plate XII, fig 17) seemed to have been torn from its fastening during the traction exerted in the dissection process, and some of the black spores did not show any contact with the gut-tissue. These twelve 'black spores' had dimensions almost equal to those of twelve similar forms seen lying on oocysts in the same specimen. The latter bodies averaged 10.5 microns  $\times$  2 microns and the average size of the former was 11.0 microns  $\times$  2.3 microns. In both sets the colour was black by reflected light and dark yellow or light brown when viewed by transmitted (electric) light.

A very striking example of tracheal connections exhibited by 'black spores' was observed on the gut-wall of a specimen of *Culex fatigans*. This mosquito was found to harbour oocyst capsules and gland sporozoites. A single body recognizable as a typical 'black spore' of the usual dark brown variety (Type 1) was observed at first apparently isolated alongside of a tracheal tube. This latter was broken into six islands of light yellow chitinous particles of the same form but slightly larger than the 'black spore' which was nearly touching it. The interrupted spiral wrapping of the tracheal tube, resembling the torn insulation of an old electric light wire, could be traced quite easily. This was also found to connect with a fork which ran in the direction of the 'black spore' but was apparently separated by a distance of 250 microns, but by careful focussing the waxy-like tracheal continuation was seen to connect with the 'black spore' forming an intermittent line for a considerable distance on the surface of the gut-wall.

## V DISCUSSION AND SUMMARY

The points in favour of a tracheal origin of most, if not all, the bodies known as 'black spores' are —

(a) The first thing that strikes one in examining a mass of 'black spores' is its resemblance to a snarl of tracheæ. Isolated spores are very similar in shape and size to small portions of tracheæ containing air. The continuity of the spiral thread is seen to be repeatedly obscured, when chitin depositions occur as 'black spores' (*vide* Plate XI, fig 15)

(b) Connections with tracheæ have been traced entering and leaving these bodies. This is very evident in isolated specimens, such as that depicted in Plate XI, fig 13. As has been pointed out by Sharp (1910), all the organs are abundantly supplied with a capillary tracheal network and in some cases the tubes enter the substance of the tissues near their termination. This has an obvious bearing in accounting for the presence of chitinized tracheæ embedded in the capsules of gut oocysts. It would explain the presence of isolated fragments of tracheal material in the muscular tissue of the mosquito's thorax.

(c) These bodies have been treated with caustic potash for varying periods of 10 to 96 hours, and, as already mentioned by Brug, Walch and Daniels, this chemical seems to have no solvent action on them. Indeed, they have not altered in form and colour when kept in a mounting medium of varying strengths of formaldehyde solution for fully ten months, although the tracheal relations, evident in fresh specimens, have become difficult or impossible to trace in such specimens. In this connection Miall and Denny (1866) have indicated the great chemical stability of the chitinous lining of the tracheal tubes. They found, as previously mentioned, that the substance resisted the action of water for fully a year.

This peculiarity gives strong support to the view that these bodies are chitinous (e.g., 'chitin corpuscles' of Brug). One knows that true chitin is mainly, if not entirely, of ectodermal origin and, if one considers the development of the internal structures of the mosquito, the only ectodermal tissue present are the tracheæ. This fact supports the view that such chitinous bodies could only be formed by the chitinogenous cells that are present in the tracheal walls.

(d) In certain specimens a definite lumen appears to be present inside the spores, continuous with, but narrower than, the lumen of the tracheæ from which the body appeared to be derived. The thickness of the 'black spores' averages 2.5 microns, and one can readily see how similar this measure is to that of the smaller tracheæ (*vide* Plate XII, fig 18).

(e) In many specimens the 'black spores' appear to be slightly thicker than the tracheæ from which they are derived. This one would expect if the bodies are the result of an excessive production of chitin by the layer of chitinogenous cells.

(f) The varying grades of intensity of colour from yellow to black seem to be dependent upon the thickness of the chitin which has been laid down, for,

as mentioned in the description of the histology of the tracheæ, the chitin as seen in thin layers is yellow while thicker layers are darker and may be quite black. In some specimens areas of distinct pale yellow chitin could be made out along an apparently normal tracheal tube, which may represent a very early stage of the 'black spores'. These thin sheets of amber yellow are probably the constituents of the 'chitinized sporozoites' referred to by Ruge, who has described transitional forms between 'black spores' and brown sporozoites in the oocyst.

(g) Certain appearances have been seen suggesting that some of the 'black spores' might be present as actual solid bodies *within* the lumen of the tracheæ, but no definite evidence could be obtained to confirm this, and from the normal histology of the trachea, one would not expect solid chitinogenous masses to occur free in this position, although one could understand a thickening of the wall sufficient to cause an apparent occlusion of the lumen of the tube. Indeed, in some cases 'black spores' were seen in which although a continuity of the lumen of the tracheal tube could be seen, it appeared to be definitely constricted by the thickness of the tracheal wall. The exact position of such apparently intra-tracheal bodies could only be definitely settled by sections, but unfortunately this was not done.

In one example of *C. fatigans*, where oocysts were also present, there was noticed a concentration of clear tracheal threads on the surface of the gut-wall with, here and there, individual 'black spores' apparently superimposed. These dark bodies, when viewed under high power, seemed to lie within the spiral thread of the tracheal tube.

Although the present writer has no desire to magnify the importance of the problem of the 'black spores,' he wishes to indicate that it is unwise to minimize the importance of a knowledge of the true nature of these structures. He wishes merely to revive an interest in bodies which were regarded by various authors as of almost equal significance to oocysts or sporozoites in indicating malarial infection in the mosquito. An examination of the copious literature makes one realize that an investigation is opportune in order to correct the accumulation of mis-statements concerning the significance of these structures. As Hindle (1914) has clearly set forth, the frequently expressed suggestion of other channels of infection, besides that of the mosquito bite, gave a distorted value to the presence of 'black spores' within the parasitic cysts.

One of the practical points to consider, in regarding the 'black spores' of Ross as specific entities, is to guard against applying their presence as a test of any pathogenic relationship to malaria, especially in the absence of parasites.

An effort has been made in this paper to indicate the error of associating 'black spores' with malaria parasites and infectivity. Rather, I have attempted to show that these bodies occur both in infected and uninfected mosquitoes, that they are not always found in oocysts or in company with sporozoites, that they are not either involution or developmental forms of the malaria parasite. Their chitinous nature provides an explanation for their colour and consistency, their tracheal origin for their form and size. One is impressed by the general



uniformity of thickness of the tubular 'black spores,' coincidentally the majority of them are just double the thickness of sporozoites. This is explained by the following facts—These bodies are formed usually in the terminal tubes of the tracheæ at the juncture where they finally anastomose into the tracheoles, but at a point where the tracheæ still retain their spiral tæmdia. It was observed that the tracheæ at this location measure from 2 to 3 microns, and the 'black spores,' whether connected with tracheæ or without any obvious connection, measure on the average 2.5 microns.

It is interesting to note that the idea of association of 'black spores' with tracheal depositions in the mosquito is not entirely an innovation. And I am glad to waive the distinction of priority in the discovery of the nature of these chitin-bodies. Following the studies and general conclusions drawn, the writer was fortunate in obtaining a copy of Walch's original paper on 'black spores.' This paper of Walch (1922) contained the report of the discussion on it by members attending the 4th Congress of the Far Eastern Association of Tropical Medicine. This section of the original paper was published in English and contains some very pertinent and significant references to the nature of the 'black spores' of Ross.

In the discussion Dr. Schuurmans Stekhoven remarked, that he was impressed by a photomicrograph exhibited by Dr. Walch in which the banana-like 'black spores' were shown in the neighbourhood of the tracheæ and in or upon the walls of the tracheal system. The illustration suggested to him 'that these black corpuscles grew in connection with the trachean layer.' The interrogator inquired if Walch had found 'other accidental black formations in other places in the body of the mosquito' where these corpuscles may have originated in response to a stimulus influencing chitin-forming cells on the part of the parasites. In answer to Dr. Schuurmans Stekhoven, Walch is quoted as saying 'I had already read in the publication of Brug that he thought it possible that the tracheæ should be of some importance to the formation of chitin in cysts. Therefore, I have studied this point closely, but I cannot say I saw more trachea in the neighbourhood of cysts which contained chitin than in those that involuted in the ordinary manner.'

The writer desires to draw attention to a misconception of views as regards 'black spores' and a theory entertained by many writers on malaria parasitology. It is the oft-repeated explanation of hyper-parasitism to account for the invasion of the malarial oocyst by other parasites to render the function of the malaria parasite impotent. In nearly every reference where this delectable hypothesis is mentioned there appears a decided confusion between the conventional 'black spores' of Ross and the microsporidian of the genus *Nosema*, which may possibly prey on the oocyst of the mosquito and destroy it. In this connection the similarity of these bodies has been ably controverted by Wenyon (1926), who succinctly points out 'That the "black spores" are spores of a microsporidian cannot be entertained, as they do not bear any resemblance to these.'

Manson-Balir (1925) indicates that the 'black spores' of Ross and *Nosema* are distinct inasmuch as the latter is a microsporidian, which may occur in the mosquito irrespective of the malaria parasite. These interpretations are undoubtedly correct and should, I think, be universally adopted.

### CONCLUSIONS

The evidence brought forward in this paper leads to the following conclusions —

(a) The 'black spores' originally described by Ross are not parasites nor are they any stage in the development of the malaria parasite in the mosquito.

(b) The hypothesis of Grassi and other workers that these bodies are concerned with 'regression or involution' processes of the oocyst is not confirmed, nor that lowered temperatures favour the production of these bodies.

(c) The 'black spores' have been found during experiments with avian malaria in uninfected mosquitoes as well as in mosquitoes harbouring the parasites of malaria.

(d) In addition, the 'black spores' have been observed in the following insects, undeniably unassociated with malaria —

(1) In freshly-emerged, unfed, laboratory-bred females and males of *Anopheles* and *Culex*.

(2) In house-flies, *Musca* sp.

(e) The 'black spores' have been found to react to the test for chitin.

(f) Most, if not all, 'black spores' appear to be chitinous thickenings of tracheal tubes. This conclusion forms a rational and simple explanation of the nature and distribution of the various types of the bodies described.

### ACKNOWLEDGMENTS

My grateful acknowledgments are due to Major J. A. Sinton, V.C., O.B.E., I.M.S., Director of the Malaria Survey of India, for furnishing me with the literature required for this paper and his translations from the Italian and French articles. Also for his encouragement in the interpretation of the nature of the 'black spore'. He was kind enough to examine much of the material used in this research and to confirm the fact that many of these bodies were intimately associated with the tracheæ.

I am indebted to Captain P. J. Bairaud, RES, F.Z.S., F.L.S., Medical Entomologist to the Malaria Survey of India, for the identifications of the mosquitoes examined in the course of this investigation. Likewise for his aid in the study of anatomical details.

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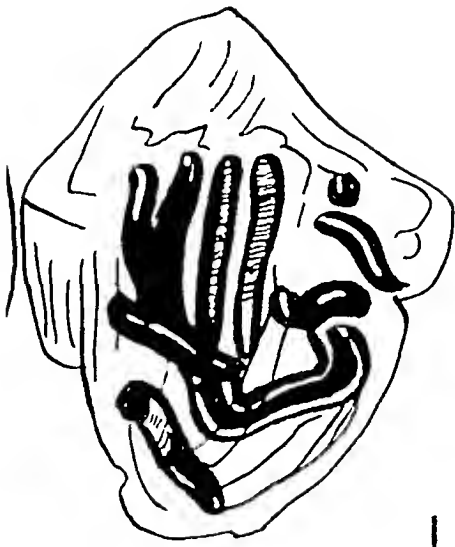
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\* James (1928) demonstrates the presence of the brown sausage-shaped bodies scattered through the thorax and salivary glands and the gut-wall in 78 infected specimens of *A. maculipennis* out of 2,300 dissected. These 'black spores' were observed invariably in the presence of zygotes or sporozoites. James tabulates the monthly distribution of the occurrence of these bodies through the years 1925, 1926, and 1927. He indicates clearly the direct relationship to malaria parasites inasmuch as the 'spores' were never found in the absence of parasites of human malaria nor during the period when these forms were undergoing development in the mosquito up to the 7th day.



PLATE IX



2



3



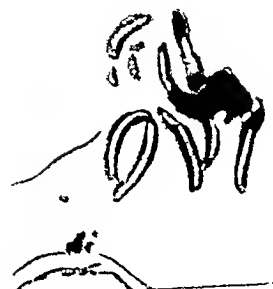
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8

## EXPLANATION OF PLATE IX

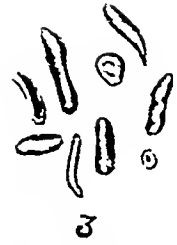
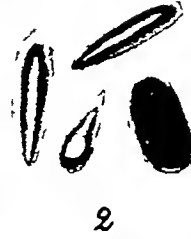
- Fig 1 Copied from Ross (1923) 'Memoirs,' page 115 (Plate II, fig 38)  
The capsule of an empty zygote containing 'black spores' This is an unretouched photograph of Ross's sketch showing 9 or 10 dark tubular forms of the size of thickened sporozoites resting upon the empty capsule of a malaria oocyst These structures are strikingly similar to tracheæ Two are shown with tænidia-like lines within dark-lined tubular forms, one appears as a continuous unjointed tube with a black core running throughout its length, and an individual rounded body may be interpreted as the visible end of a chitinized tracheal tube
- „ 2 Copied  $\times 9/10$  from Grassi (1901) (Fig 22 and 23)  
Brown bodies found unattached in the coelomic cavity of an anopheline mosquito infected 13 days Mounted in glycerine formalin solution These bodies are regarded by me as fragments of the tracheæ detached in the process of dissection
- „ 3 Copied from Grassi (1901) (Fig 20)  
Capsule of an oocyst of *Plasmodium falciparum* (11th day of incubation) containing typical 'brown bodies'  
In the unretouched copy of Grassi's illustration are 19 more or less banana-shaped structures, distinctly with hollow or lighter interiors In some of these forms one can detect spiral lines very suggestive of portions of tracheal tubes The two round bodies may be cross sections of exposed ends of tracheæ which have penetrated the oocyst
- „ 4 Copied  $\times 2$  from Walch (1922) (Fig 14) He considers this the complete enveloping of a hyphomycete in a heavy chitinous covering on the mosquito's gut The separated forms he refers to as chitinized mycelium These forms are discussed in this connection because Walch regarded the general condition of chitinization in a parasite as a reaction to a defence against invasion of a foreign body, such as that of the malaria organism in the mosquito  
I have observed quite a similar appearance in a bifid branched tracheal tube having a transparent envelope formed about a dense chitinous interior
- Figs 5 and 6 Copied  $\times 2$  from Walch (1922) (Figs 8 and 9) Brown curved bodies found in the region of the salivary gland and of oocyst on gut The general tendency to burrow into the soft tissue is here illustrated These forms were tested by Walch who found them to be mainly chitinous
- Fig 7 Copied  $\times 2$  from Ruge (1903) (Fig 14)  
A mass of 'Ross's spores' on the gut-wall of *Culex pipiens* supposed to be developed from sporozoites Ruge defines these as S- or comma-shaped, yellow to dark brown, and as long as or longer than sporozoites, being double in width
- „ 8 Copied  $\times 2$  from Ruge (1903) (Fig 13  $\times 500$ ) These were found on the gut-wall of an infected *Culex pipiens* regarded by the author as transition forms between brown sporozoites and Ross's 'black spores'  
They are distinctively tubular and very suggestive of sections of torn tracheæ

## EXPLANATION OF PLATE X

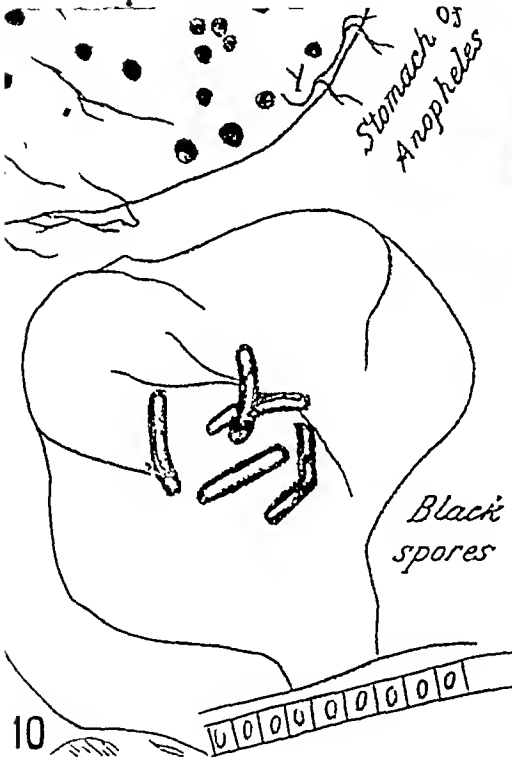
- Fig 9 Copied  $\times 1\frac{1}{2}$  from Wenyon (1926) ( $\times 1,000$ ) No 1—oocyst and 'black spores' in *A. maculipennis* infected with *Plasmodium vivax*  
Preparation of Col S P James
- No 2—isolated 'black spores' from an anopheline S P J preparation
- No 3—'black spores' from a Macedonian mosquito infected with *P. falciparum* (Col C M Wenyon's preparation)
- The general tubular structure in most of the forms shown is noteworthy Their affinity to tracheal tubes is quite suggestive They are said to be probably the result of the death and degeneration of the oocyst contents, the mosquito depositing chitinous material in them
- „ 10 Copied  $\times 2$ , Ross (1905) original reference to 'black spores' from *Journal R A M C* (1905), p 732 (Fig 20) Described as a zygote of the 6th day and later The five forms in the sketch are interpreted as 'black spores' The hollow tubular-like structure is quite distinct, their resemblance to stranded portions of tracheal tubes is suggestive
- „ 11 Photomicrograph (original) Portion of gut-wall of *Culex fatigans*  
A field of the tracheal system showing typical clumping Similar clumps commonly form masses of 'black spores'
- „ 12 Copied (enlarged) from Brug (1916) Thought to be an aggregation of 'black spores' formed from the wrinkling of the cyst-wall of an oocyst and subsequently becoming chitinized The resemblance to tracheæ is very striking
- „ 12a A group of 'black spores' 'chitin corpuscles' on the gut-wall of a *Culex pipiens* infected with *Plasmodium praecox*  
At (b) an association of the chitinized structures with the tracheæ is indicated



9



*Stomach of  
Anopheles*



*Black  
spores*



12



12 A.

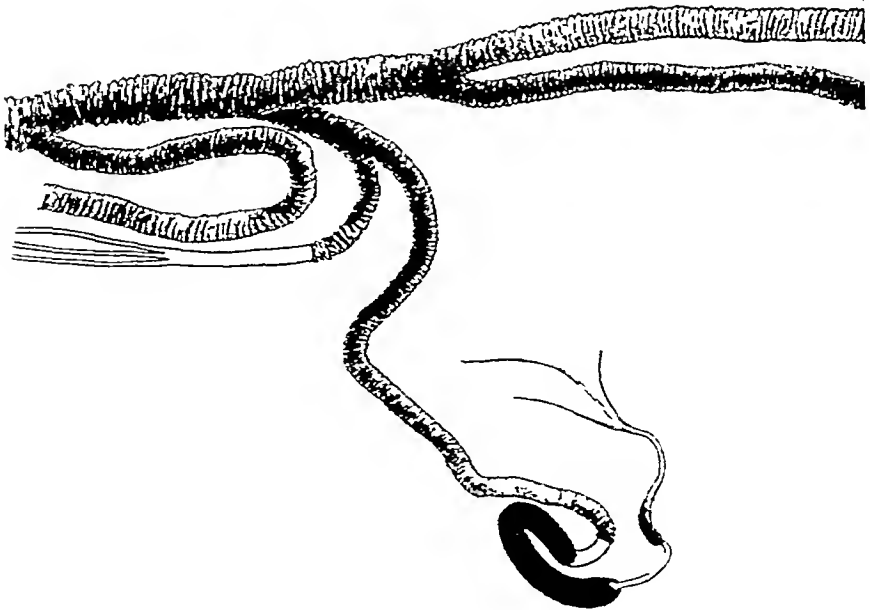


## EXPLANATION OF PLATE XI

- Fig 13 Photomicrographs (original) mass of 'black spores' on gut-wall of *Culex fatigans* infected with avian plasmodia. These bodies were found to measure actually the same length (10 microns to 12.5 microns) as the sporozoites expressed from the same specimen of mosquito.
- „ 14 Photomicrograph (original) Gut-wall of *Culex fatigans* 10th day following infective bite. Dissected specimen unstained, mounted in formalin. There are two free masses of chitin-bodies, a group of three in a half-grown oocyst and an oocyst in the segmenting stage measuring 30 microns. On the capsule of the latter appears the broken end of a tracheal tube. This was found to measure 10 microns, the same as many of the 'black spores' shown in the two masses.
- „ 15 Photomicrographs (original) Thoracic muscle close to salivary glands of *Culex fatigans*. Here were found three lines of 'black spores' and those illustrated give one the impression that they resemble the forms which Walch speculatively regards as 'migratory sporozoites which have undergone chitinization while wandering through the body-tissue'. The 'black spores' figure measured 10 to 15 microns.
- „ 16 Photomicrograph (original) Thorax of *Anopheles subpictus* showing dark brown bodies in and near a large trachea. Careful focussing revealed all but four of them surrounded by the tænidial envelope of the trachea. These 'black spores' measured about 10 microns.

## EXPLANATION OF PLATE XII

- Fig 17 Photomicrographs (original) A field on the gut-wall of a severely infected *Culex fatigans* shown in the same focus, the dorsal aspect. In this view there are 12 typical 'black spores,' six in the same focus, the remainder lost to view. Those clearly shown appear in the mass of tangled tracheæ which are apparently super-imposed. They seem to have no direct connection with the gut-wall tissue, which in other regions is invaded by numerous oocysts, the capsules of which were very prominent. On the muscular gut-wall in this mosquito, the tracheal system appears unusually well supplied with tracheal tubes which give the impression of concentration in the area selected for illustration.
- „ 18 *Camera lucida drawing*  
 From the gut-wall of an uninfected (wild) *Anopheles subpictus*. Specimen viewed by oil immersion objective and  $\times 15$  ocular. There are shown several branched tracheal tubes merging into tracheoles with a small and a large 'black spore,' their sizes limited by the varying diameter of the single tracheal tube in which apparently the chitin bodies were deposited. In the tube of smaller calibre there were no tænidia visible, whilst the deposition of the chitinous mass at the wider portion seemed to have obliterated the spiral markings of the trachea. The larger 'black spore,' measuring 20 microns long by 2.3 microns in width, is typical in form and appearance to the majority of similar bodies observed in the course of these studies. The preparation and the drawing of this specimen were both very kindly made by Dr I. M. Puri, M.Sc., Ph.D.





# PERSISTENT THYMUS \*

BY

T BHASKARA MENON, M D, M R C P,  
*Department of Pathology, Medical College, Madras*

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THE association of a persistent thymus with Kopp's thymic asthma, thymic stridor and so-called thymic death is a subject which has been engaging attention during the last half century

I shall only touch on the controversy ranging between Friedleben and Platauf on the one hand and Kopp and his school on the other as to whether an enlarged thymus can cause a tracheo-stenosis and sudden death. That there is some association between an enlarged or a persistent thymus and sudden death is a fact which I believe few would attempt to deny, but before attributing casual factors to the thymus in such conditions I would like to draw your attention to certain interesting features that have come to my notice with sufficient persistence to deserve fuller investigation. I refer to the presence of a persistent or enlarged thymus where death has been sudden, i.e., in about 24 hours.

This association of a persistent thymus with medico-legal deaths I shall show in the following series of cases though unfortunately the weight of the organ has not been recorded, since it was an accidental finding —

No of P M	Age	Condition of thymus	Rapidity of death	Cause of death	Nature of accident
P M 2105 29-5-27	10	Hyperplasia	Few hours	Fracture skull	Bus accident
P M 2109 7-6-27	35	Persistence	1 day	Fracture femur gangrene	Do
P M 2121 29-6-27	30	Do	Few hours	Fracture skull	Do
P M 2126 7-7-27	33	Do	1 day	Gun-shot wound abdomen	Gun-shot.
P M 2142 23-7-27	50	Do	Few hours	Multiple fracture rib	Bus accident
P M 2143 24-7-27	30	Do	Do	Fracture skull	Do

\* Paper read before the Indian Science Congress, Madras, 1929

No of P M	Age	Condition of thymus	Rapidity of death	Cause of death	Nature of accident
P M 2171 14-9-27	50	Persistence	1 day	Ventricular hemorrhage	Bus accident
P M 2179 25-9-27	30	Do	Few hours	Post-operative shock	Post-operative
P M 2178 23-9-27	40	No thymus	12 days	Compound fracture pneumonia	Car accident
P M 2188 13-10-27	25	Persistence	1 day	Fracture skull	Bus accident
P M 2195 23-10-27	12	Hyperplasia	Do	Do	Car accident
P M 2194 23-10-27	2½	Do	Do	Multiple fracture	Bus accident
P M 2271 28-2-28	60	Persistence	Few hours	Do	Do
P M 2272 2-3-28	25	39.5 grammes	Do	Do	Do
P M 2273 3-3-28	20	28.6 grammes	Do	Stab wound	
P M 2275 7-3-28	30	Hyperplasia	1 day	Fracture skull	Bus accident
P M 2276 7-3-28	54	Persistence	Do	Do	Do
P M 2291 24-3-28	20	Do	Do	Do	Do
P M 2292 25-3-28	10	Hyperplasia	Do		
P M 2295 31-3-28	30	Persistence	Do	Multiple fracture	Bus accident
P M 2296 1-4-28	7	Hyperplasia	Do	Multiple injuries	
P M 2304 12-4-28	17	Persistence	2 days	Multiple fracture	Railway accident
P M 2313 7-5-28	22	Do	Few hours	Stab wound	
P M 2314 7-5-28	15	Do	1 day	Rupture stomach	Bus accident
P M 2319 18-5-28	23	Do	Do	Corrosive poisoning	
P M 2339 30-6-28	70	Do	Do	Fracture skull	Bus accident
P M 2354 31-7-28	30	Do	Few hours	Acute heart failure	



From an analysis of the above table it will be at once apparent that the relation of the persistent thymus to sudden death seems independent of the cause of the death and seems to be more associated with rapidity of it. The idea that emerges is that a persistent enlarged thymus is found in a large proportion of cases of sudden death whether the death is due to gun-shot wound, fractures of the skull from bus accidents, multiple fracture of the ribs, subdural hæmorrhage, or chloroform anæsthesia.

The studies of Hammar and of Schridde indicate that the thymus is an organ which persists throughout life and that the term persistent thymus itself is a misnomer. The following table cited by Marine shows the variations in weight of the organ with age as recorded by Hammar and Schridde —

Age	WEIGHTS OF THYMUS IN GRAMMS	
	Hammar	Schridde
New born	13	13
1—5 years	23	17
6—10 years	26	20
11—15 years	38	25
16—20 years	26	20
21—25 years	25	19
26—35 years	20	14
36—45 years	16	10
46—55 years	13	7
56—65 years	16	4
66—75 years	6	3

Hammar's figures have been blamed for including mild cases of status lymphaticus and not including pathologic involution and have been taken as slightly high.

It is obvious that with an organ of which the size and weight decrease so very much after adolescence cases of so-called persistent thymus might come under one of two types, i.e., cases in which the remnants of the organ have undergone hyperplasia just before death or some time before death as a result of some metabolic disorder or those cases in which the organ persists in its original adolescent stage.

It is very difficult to make out whether the above cases are instances of reactive hyperplasia or of a real persistence in an adolescent stage.

How are we then to explain the presence of the enlarged thymus in cases of sudden death? Major Forsyth, a previous Professor of Pathology in Madras,

had himself remarked on the unusual association of these thymuses in medico-legal deaths. He has offered an ingenious explanation which is in consonance with the view of thymic death. He believed that persons with an enlarged thymus were not brisk enough to defend themselves when attacked or get out of the way of accidents. This theory, however, cannot explain a large number of medico-legal deaths, bus accidents, gun-shot wounds, etc., in which the injured persons has been in situations in which he could not possibly avoid the accident. It is surely impossible to assume that a person has this hypothetical 'thymic lethargy' when he is merely a passenger in a bus which crashes into another and several people are injured.

It may be argued that this association of a persistent thymus is only apparent and not real. For pathologists who deal with the end stages of disease generally in the aged or in middle life, when they are brought into contact with medico-legal deaths in which healthy robust individuals often very young are involved, naturally find the thymus in an unusual number of cases.

However an analysis of the above figures shows that this association is not merely apparent since a number of cases occur in which the organ has been found in a persistent stage in middle-aged and old individuals.

It must be understood that the post mortem size and weight of the organ may be no index of its condition before death. Hammar advances a theory of great interest. He believes that the thymus is never found in a normal condition in persons dead of disease, since it is this tissue which is most susceptible to all forms of disease. I would point out that the apparent persistent thymus met with in cases of sudden death would according to this view be the unaltered thymus appearing unduly large. Hammar asserts on histological grounds that there is no structural difference between the normal thymus and so-called persistent thymus of *status lymphaticus*.

Ruhrh and Dudgeon have pointed out that in cases of protracted marasmus in children there is an atrophy of the thymus. Such atrophic changes have been found in chronic tuberculosis, empyema and bronchiectasis. I have found a similar instance in a child in a case of extreme ankylostomiasis. I believe that this holds true equally well in the adult where it is quite common to find little thymic tissue in chronic wasting diseases, like tuberculosis, chronic diarrhoeas, etc. But our argument is that it is not the chronic wasting disease that interferes with the thymus, but that this organ has a tendency to atrophy with all chronic and protracted forms of tissue death.

If we take into account the relation between the thymus and the other ductless glands, we may possibly find an explanation for the condition. Marie has pointed out the regeneration of the thymus that occurs in exophthalmic goitre, but others believe that there is a separate 'Thymic Basedow's' and that primary Graves' disease does not exhibit this regeneration. However, Williamson and Pearse have drawn attention to the hypertrophy of the thyroid in many cases of *status lymphaticus*, and to the lymphocytic activity which occurs in the thymus when the thyroid is most active. They have been able to demonstrate

the presence of a thyro-thymic leash of lymphatics and hold that the stalk of the thymus takes origin from the thyroid and that masses of thymic tissue containing Hassel's corpuscles extend all the way up along the lymphatic channels to the thyroid. They advance the theory that the thymus is a reservoir of the thyroid secretion—a secretion which is, however, quite different from colloid—and they argue that the thymus does not undergo atrophy but manifests a metamorphism to a vesiculate fat stage in the adult. In attempting to explain the sudden death in goitrous conditions, however, they bring in *status lymphaticus* which was present in 90 per cent of their cases.

The evidence of a thymic hyperplasia in acute infections has been pointed out by Friedleben and in cases of diphtheria by O'Hausen as well as by Roger and Ghika who have experimentally produced it in guinea-pigs by injections of cultures of streptococci and staphylococci. Friedleben has noted atrophy in chronic infections.

The whole question has important bearings on the problems of the thymic death and *status lymphaticus*. Following the views of Kopp most writers, and even recent workers, still hold that *status lymphaticus* is sufficiently explanatory of the conditions of sudden death met with. I would point out that this is viewing the problem from a wrong angle. For, if it is proved that the condition of the thymus in *status lymphaticus* is only associated with the suddenness of the death and not in any way causal, the whole aspect of the problem would be changed. The cases reported as *status lymphaticus* would then be cases in which death was very sudden and consequently the thymus did not have time enough to change into the condition of the atrophied fat-laden organ that is met with in autopsies in cases of chronic death.

I am not here attempting to offer an explanation for these cases of sudden death without obvious lesions, but the argument that is advanced is that it is not in the thymus that we must look for the cause but elsewhere. The persistent thymus is only the result of the suddenness of the death, in the sense that the death was too sudden for the thymus to undergo changes.

If we are to accept this theory, the behaviour of the thymus becomes clear. Thus in acute infection the basal metabolic rate would be high indicating an increased activity of the thyroid and consequently of the thymus, and the thymus would be in a state of hyperplasia if death were to occur rapidly. In chronic wasting diseases, the basal metabolic rate would be low indicating an exhaustion of the thyroid apparatus and consequently the thymus would be in an atrophied state at the time of death. This would explain the so-called physiological atrophy that is met with in autopsies in general. If we come to explain the occurrence of the persistent thymus in sudden death due to accidents, the view of the persistence of the normal organ which had no time to undergo atrophy would meet the case. In those cases of sudden death in which no cause can be found, the explanation of the death would have to be sought for in the heart muscle or elsewhere, for it is surely not rational to lay the blame on an organ which shows a similar change in hosts of other conditions in which it could possibly have no

causal bearing Pathologists will no doubt agree that very little thymic tissue is usually encountered in hospital autopsies except in children I, therefore, venture to put forward the theory that the association of a persistent thymus with medico-legal deaths is due to the suddenness of the death The thymus is an organ which is extremely susceptible to all deleterious agents and is probably the first to undergo involutionary changes in illnesses ending in death As already explained this is made apparent from its inter-relation with the thyroid If this is proved it follows that in sudden and acute forms of death the thymus is caught unawares—so to say—and had no time to exhaust itself and change to the atrophied fat-laden organ that is so usually encountered This view also brings us into line with Hammar's view of a 'physiological' persistence and affords explanation for his normal weights which have been criticized as too high

### SUMMARY

(1) The association of a persistent thymus with medico-legal deaths due to various causes is emphasized

(2) The theory is put forward that this is due to the suddenness of the death, death being so sudden that the thymus had not time enough to undergo atrophy or fat metamorphosis

(3) It is advanced that the thymus has no causal bearing in these sudden deaths

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# SOME OBSERVATIONS ON THE PROCESS OF MAKING GHEE AND ITS EFFECT ON THE LEGAL STANDARDS

BY

LIEUT -COL A D STEWART, M B, FRCSE, DPH, DTM & H, IMS,  
*Professor of Hygiene,*

AND

N L BANERJEA, M Sc (Cal), AIC,  
*Assistant Professor of Public Health Laboratory Practice,  
Calcutta School of Tropical Medicine and Hygiene*

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IN India clarified butter fat is sold under the name of 'Ghee,' because butter does not keep well in tropical conditions. In making ghee, the curd and water are eliminated. There are several methods of making ghee from butter, involving factors which might alter its analytical figures, thus ghee might be prepared from butter churned from raw milk or from 'dahi' made of boiled milk with a 'starter'. Then the 'dahi' might be kept indefinitely before being churned for butter from which the ghee is finally made. To make ghee, butter is melted and then clarified by 'heating' at varying temperatures from 100°C to 180°C (B P of ghee). The question arises whether these varieties in preparation affect the analytical constants. Thus Dr Brahmachari(1) in his study of constants of pure cow's ghee states 'methods of preparation leave room for introduction of fallacies'. He prepared his ghee consistently according to one method, viz, by melting and heating butter over a direct flame nearly to the boiling point of the fat. He also prepared his butter from 'dahi' made from boiled milk with a starter. We have, however, found that none of these procedures need be strictly followed in making ghee in so far as the analytical constants like butyro-refractometer reading and Reichert Wollny values are concerned.

## EXPERIMENT No 1

A sample of pure cow's milk was taken and churned in a Daisy churner in a raw state until butter was obtained. This butter was divided into two portions  
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One portion was kept on a water bath for two days until all the moisture was driven out

Clear fat obtained after separating curd gave a Reichert Wollny value of 22.9 and butyro-refractometer of 43.0

Another portion of the butter was heated to about 160°C when the casein was just charred. The filtered fat gave a R. W. value of 22.9 and butyro-refractometer reading of 43.0 as before. The temperature, therefore, to which butter may be heated to obtain 'ghee' does not affect these values.

Higher temperatures are not used in the preparation of ghee as its taste and smell are altered.

Dahi was prepared from the same sample of milk, sterilized by boiling and inoculated with a starter, the butter obtained by churning was next divided into two portions, each portion being treated in the same way as before, as regards temperature, to obtain ghee. The R. W. values were found to be 22.9 and 23.2 respectively and the butyro-refractometer reading in both was 43.0. The discrepancy between the R. W. values falls within the range of experimental error.

It is thus evident that ghee whether obtained from unboiled raw milk or from dahi, preserves its analytical values unaltered. Incidentally, it has been noticed that ghee prepared from dahi possesses the characteristic flavour, while the one made from unboiled milk has none.

## EXPERIMENT No. 2

*Variation of analytical constants, if any, of ghee with the change in the lactic acid content of dahi due to storage*—A sterilized sample of pure cow's milk was divided into two portions. Each portion was kept in a separate vessel and inoculated with a starter. The content of one vessel was kept overnight and the other was kept for seven days. Ghee was prepared from butter got by churning, by heating it to a temperature between 120°C and 130°C for half an hour to remove water. The characteristic ghee-like aroma was then found to develop and the casein assumed the chestnut brown colour, imparting a slight tinge of yellow to the ghee due to the caramelization. The heating should not be continued over half an hour, otherwise much of the aroma is lost and replaced by the odour of burnt casein.

The following are the results—

	Per cent of lactic acid	R. W. figure	Butyro-refractometer reading at 40°
Dahi of 12 hours' standing	1.45	26.6	41.9
Dahi of 7 days' standing	2.13	26.6	41.9

It will be seen from the results that the analytical values are entirely unaffected even if the dahi is kept for seven days when the lactic acid developed.

was one and a half times that present in the other dahi. Dahi kept longer begins to decompose.

It was found that the aroma present in the ghee prepared through the medium of dahi was absent in ghee prepared directly from unboiled milk, further, ghee made by melting and heating the butter at an ordinary temperature of water bath, developed only slight aroma and the aroma increases as the lactic acid content of the dahi increases on keeping. It would seem that the aroma associated with lactic acid is possibly due to esterification of either the acid or its degradation products, such as propionic or butyric acid with alcohol which is very likely to be formed as a by-product in the fermentation process. The essential condition for esterification is complete elimination of water which cannot, however, take place below  $100^{\circ}\text{C}$  (temperature of water bath) as was observed in our experiment.

Hence the optimum range of temperature was found to be between  $120^{\circ}\text{C}$  and  $130^{\circ}\text{C}$ . The nature of the aroma is doubtful and needs more investigation. A similar aroma is noticed in cheese during its ripening which according to Lewkowitsch(2) is due to a particular bacterium present in the starter. Allen(3) says that it is due to an amino-acid formed from casein.

Another point worth investigating is the loss of vitamin that may take place in ghee while it is being made from butter by heating it to a temperature between  $120^{\circ}\text{C}$  and  $130^{\circ}\text{C}$ . It is difficult to prepare ghee below  $120^{\circ}\text{C}$  as at lower temperatures expulsion of moisture would take a longer time which might prove injurious to the vitamins and also the aroma would not be fully developed. The elimination of moisture is a *sine qua non* for insuring freedom of ghee from rancidity. It is a question whether ghee may retain vitamin A if it is heated at a temperature of  $120^{\circ}\text{C}$  to  $130^{\circ}\text{C}$  which is the optimum both for its aroma and keeping quality. We know that vitamin A is somewhat thermostable(4) and also that butter requires only half an hour's heating at this temperature for preparing ghee.

If it is found by biological experiment that ghee thus prepared loses all its vitamin A, the whole mode of its indigenous preparation should be changed. The moisture would have to be driven out at a temperature between  $60^{\circ}\text{C}$  and  $70^{\circ}\text{C}$  which can only be done under vacuum. The oxidation process which is the main cause of deterioration of vitamin A in ghee will thereby be retarded, but the aroma may not develop at this low temperature. As esterification is known to take place even at ordinary temperature, it is worth finding out if the aroma will develop later if the ghee is kept in a dry state.

### EXPERIMENT No 3

*Effect of the increase of lactic acid content of dahi on its yield of ghee—*

A much larger yield of butter is got when dahi is kept for seven days as compared with the other dahi. The lactic acid developed is also much greater. There is obviously some relationship between the amount of lactic acid and the yield of ghee. The reason for this was studied in the alteration of surface

tension that takes place. Samples of sera were collected from dahi kept for varying periods and their surface tensions estimated by means of Dr Du Nouy's tensiometer (5). This instrument measures directly in dynes per cm the force exerted by the surface film of the liquid under test upon a platinum ring when pulled away from the liquid in which it has been dipped. This method is free from complications, e.g., contact angle, etc., in which other methods are involved.

Results —

	Per cent of lactic acid	Surface tension in dynes per cm	Yield of ghee
Milk		49.4	11 gms
Dahi of 1 day's standing	1.04	47.0	13 „
Dahi of 3 days' standing	1.45	45.2	14 „
Dahi of 5 days' standing	1.65	42.3	16 „

Time of churning two hours

In case of the dahi kept for five days practically all the butter was obtained within 15 minutes, in other cases two hours was necessary. The increase in the lactic acid content in the dahi is, therefore, apparently responsible both for the greater yield of fat and for the shortening of the time of churning necessary.

Milk is a stable emulsion which is of oil-in-water type where droplets of fat are dispersed in water, whereas butter is of opposite type (water-in-oil), being a solid emulsion of fat with fat as the continuous medium in which drops of water are dispersed. By churning and by the development of lactic acid the phases are reversed, the oil-in-water type being changed into the water-in-oil type. The formation of lactic acid acts as an auxiliary to the process of churning. The increase in the lactic acid content is accompanied by a lowering of the surface tension of the serum. Gibbs (6) states that any substance that lowers surface tension must concentrate at the surfaces of contact between two liquids. In this instance lactic acid while it lowers the surface tension of the milk serum must have a greater concentration at the interfaces between fat and the serum in the dahi than in the serum itself and the attraction intensity between the particles of fat, therefore, increases\*. In milk there exists an adsorption layer of some kind around the fat globules which prevent them from coalescing (7). During churning this layer is thinned out by the impact of the various globules permitting coalescence into nuclei which gradually grow. When lactic acid increases the thinning out of the adsorption layer is hastened by the

\*The acids tend to break the emulsion which separates into oil and water probably by some action on protective (emulsifying) agent present, e.g., casein. Hence casein is coagulated and precipitated and clots of this may be found in the butter mass [*vide* Reports on colloidal chemistry, its general and industrial applications (1921), 2nd part, p. 100].



increase in the attraction intensity of the fat globules with the result that the growth of nuclei and visibility of butter particles take place much more quickly. The experiment, therefore, justifies the manufacture of ghee from dahi, instead of from milk.

Indigenous manufacturers also make ghee from the skin which forms on milk when it is left to cool. They accumulate these skins and inoculate them with a starter. The collected products are then churned into butter. The manufacturers have found that this method of manufacture gives more aroma and increased yield, which belief is supported by scientific evidence.

When milk is boiled, albumin present in colloidal state passes out of the solution to the surface. Particles of this dissolved colloid conglomerate to give rise to a membrane or skin at the surface. Ramsden(8) found that all albumins in solution may form such a skin at the still surface of their solution as a great lowering of surface tension takes place and the colloid passes into the surface layer through adsorption. This process is irreversible. When albumin, which acts as a protective colloid to casein, goes out of solution, that portion of casein which was held bound by the former cannot remain in solution any longer, but is precipitated and carried to the surface along with albumin. Since casein and fat are inseparable unless the former is dissolved, the greater the amount of casein thus precipitated, the larger is the amount of fat carried to the surface. These skins, therefore, will yield more butter when they are subsequently macerated with water and churned. They will also give butter more quickly on account of the development of lactic acid in them.

*Effect of the fat content of individual cow's milk on its legal standards —* Buffalo milk which has a high fat content also gives fat having a high Reichert Wollny value (30 is the minimum value according to Bengal Food Adulteration Act), while cow milk with a comparatively low fat content gives fat having a low Reichert Wollny value (24 is the minimum value according to the Act). It might be thought that in individual cow's milk the R W value of ghee prepared therefore varies directly as the initial fat content. Accordingly we made a study of the relation between the percentage of fat and the R W value on twelve samples of cow's milk. The results are collated in the following table —

	Per cent of fat	R W value	Butyro-refractometer reading at 40°C
1	3.5	22.9	43.0
2	4.0	23.9	42.0
3	4.0	24.0	43.7
4	4.3	23.5	44.9
5	4.8	25.8	42.5
6	4.8	23.4	42.0
7	5.2	22.9	42.0
8	5.3	22.4	43.2
9	5.4	20.8	43.7
10	5.5	20.4	44.1
11	5.6	26.6	41.9
12	5.7	25.4	42.3

No definite relation between R W value and percentage of fat can be made out from these results. The low fat content is, however, accompanied by low R W value, while the high fat content is not, as a rule, associated with the high R W value. For instance in No 8 and No 9 although the percentage of fat is fairly high, the R W value is abnormally low.

No correlation is evident between the percentage of fat and refractometer reading.

It is interesting to make a note of these results in the light of the current standard prescribed by the Bengal Food Adulteration Act which lays down 24 as the minimum figure for R W value and the limit 40—42 for butyro-refractometer reading at 40°C.

#### SUMMARY AND CONCLUSION

Ghee whether prepared from butter churned from unboiled milk or from dahi made of boiled milk which has been kept for an indefinite period possesses the same R W value and butyro-refractometer reading. These figures remain unaffected if the ghee is made by heating butter to different degrees of temperature for clarification.

The distinctive aroma is present only in ghee prepared from dahi. On keeping dahi its lactic acid content increases with increased yield of ghee accompanied by the simultaneous decrease of surface tension of its serum. This decrease of surface tension enhances the effect of lactic acid which is responsible for the increased yield of ghee.

From the analogy that buffalo milk with its higher fat content possesses a higher R W value than the cow's milk, a relation was thought to exist between the fat content of individual cow's milk and its R W value. Twelve samples of such milk were examined and no such relation was found.

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# ON THE 'CARRIER' PROBLEM OF CHOLERA

BY

SARANJAM KHAN, B Sc, M B, B S, D P H, D T M & H

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Nor very long ago, that is to say, in the year 1920, Jorge Ricardo exclaimed, 'Happy the man who shall give us the desired solution of the problem of the carrier of vibrios'(1) The desired solution has not been forthcoming ever since the discovery of the *V cholerae* in 1883 by Robert Koch, who, in spite of his classical work on cholera, gave us no solution of the carrier problem I am not aware of any work of importance that has been done on the subject during the few years that have passed since Jorge Ricardo made the above statement On the other hand, however, the textbooks' descriptions of the subject are quite clear and leave no room for doubt in the reader's mind The result of this teaching is that the general practitioner now believes that there is exactly the same kind of 'carrier' state of cholera as there is of typhoid fever This is due in a large measure to the manner in which research workers have handled this problem It was quite natural to be influenced by the solution of the apparently similar problem presented by typhoid fever A search was, therefore, made to discover the same kind of 'chronic carriers' in cholera as were undoubtedly found in typhoid fever It appears to me that in the endeavour to do so, the analogy of typhoid fever was carried too far

## *Infection of the gall bladder is no proof of the 'chronic carrier' state*

Greig (1913) (2) has stated that it is the infected gall bladder which is responsible for the production of the 'chronic carrier' state in cholera as well as in typhoid fever Thus, 'in my research on enteric fever in India I carried out a number of daily bacteriological observations on enteric convalescents and found that a certain number of them continued to excrete the *B typhosus* intermittently for prolonged periods In the case of cholera I find that although in the majority of cases the excretion appears to cease very shortly after the acute attack, yet in 3 cases out of 11 examined daily for a considerable period the cholera vibrio was found at intervals in the stools for longer periods'(2) In another contribution he says, 'As I have shown the comma bacillus in about one-third of the cases

of cholera gains an entrance to the gall bladder, where it finds all the conditions favourable for its prolonged life. Such individuals are especially dangerous because they are reservoirs of the cholera virus, and they bear the same relation to cholera as the big game of Africa do to Nagana. Now, one is quite justified in saying that in a fairly large number of cases the cholera vibrio has been recovered from the bile of patients who have died of cholera. Goulter (1915) (3) reports the result of 226 autopsies. The cholera vibrio was found in 17 of the cases on bacteriological examination of the bile. Crowell and Johnston (1917) (4) investigated 212 cases of cholera and found the vibrio in the bile of 65.2 per cent and only in the bile in 5.7 per cent of these cases. In 32 cholera 'carriers' detected after death, the cholera vibrio was found in the bile in 75 per cent and only in the bile in 43.7 per cent.

As has been said before, the cholera vibrio may invade the gall bladder in a certain number of cholera cases. But to accept this observation as the sole or the main proof of the 'chronic carrier' state in cholera is not warranted by facts. It is not possible to agree with Greig who on simply finding the vibrio in the bile and the gall bladder of fatal cases concluded that such cases were permanent 'carriers' having the same relation to cholera as the big game of Africa to Nagana. It is established beyond doubt that the *V. cholerae* invades the mucous membrane of the small intestine and is present there in such large numbers that it would be exceptional for the gall bladder to escape infection by direct extension from the intestinal canal. As Greig and others worked with cases who had died of cholera, it does not follow that the infected state of the gall bladder, found by them, was proof of the 'chronic carrier' state. Inflammation of the bladder in these cases was found so rarely that it could be easily explained as having been due to some other cause than an infection by vibrios. Greig examined the bile of 271 cases and found vibrios to be present in 80. Of the 80 positive cases, the gall bladder was found to be healthy in 68.

In this connection it will be more interesting to know how long after an attack of cholera does the vibrio persist in the gall bladder, as compared with the intestinal mucosa. As no information is available on this point, it is reasonable to think that the source of the vibrios found in the stools of convalescents can as well be the intestinal mucosa as the gall bladder. Cantaczene and Marie (1919) (5) have shown that by whatever route the cholera vibrio enters the body, it makes for the intestines and always finally reaches the walls of the small gut. One is struck at autopsies of acute cholera cases, by the fact that vibrios are often rare in the contents of the small intestine, but if one investigates the mucous membrane deprived of its epithelium, the vibrio is encountered there in extraordinary large numbers.

There is no justification for stating that the two diseases behave alike with respect to the carrier state. In typhoid fever the bacillus persists in the gall bladder and is passed in the stools possibly for a number of years, but nothing like this happens in cholera. In connection with an epidemic of typhoid fever in Montreal in March 1927, Boucher (1927) (6) cites a case of a man who was a

'carrier' for 20 years In cholera the gall bladder owing to its connection with the gut receives its share of the vibrios infecting the intestinal mucous membrane, or at most the infection of the gall bladder is but a temporary and transient affair

*Patients are free of the vibrios within a few days*

It must be emphasized that cholera is an acute disease of short duration The *V. cholera* is passed in exceptionally large numbers during the first few days of the disease After this in the majority of cases the vibrio is no longer to be found in the stools Wesskopf and Herschmann (1915)(7) examined 247 cases of cholera in Slavonia and found that 80 per cent were free of the vibrio at the end of the illness Levi Dell A Vida (1916)(8) reported on 11,755 stools examinations with the result that 93 per cent of the cases and 99 per cent of the contacts were free of the vibrios within 14 days In Conseil's series (1912)(9), the vibrios usually disappeared by the 9th or 10th day Galambos (1916)(10) reports that out of the 89 cases of cholera except in 2 or 3 cases the vibrios disappeared from the stools in 7 to 8 days, in the exceptions they were found for 2 to 3 weeks Similar results can be quoted from other sources to show that the majority of cholera cases are free of the vibrios within the first few days of the disease

During the year 1928 (April to September), 152 cases were admitted into the Infectious Diseases Hospital at Hardwar All these cases were suffering from more or less severe vomiting and diarrhoea Clinically they all appeared to be cases of cholera We examined the stools of 134 of them and isolated vibrios from 92 cases Stools were not available or could not be examined in 18 cases Deaths and recoveries were distributed as given in the table below —

	Died	Recovered
1 Vibrio positive	41	49
2 Vibrio negative	6	36
3 Stools not available	7	1
4 Stools could not be examined	4	6
TOTAL	58	92

There were two more positive cases, the result of which as to death or recovery could not be ascertained Of the 90 cases that showed the cholera vibrio in their stools 41 died, most of them, i.e., 36 died within 5 days of the onset of disease, the longest surviving up to the 9th day By onset is meant here the fully developed disease of sufficient severity to prevent moving about Again the

majority, i.e., 36 of the fatal cases passed vibrios in their stools till their death. The result of the remaining five cases is set forth in the following table —

Number of cases	Death after onset of disease	Vibrio present up to	VIBRIO NEGATIVE PERIOD	
			Total number of days	Stools examined on
1	On the 3rd day	1st day	2 days	2 days
1	On the 4th day	2nd day	2 days	1 day
1	On the 4th day	1st day	3 days	2 days
1	On the 5th day	1st day	4 days	2 days
1	On the 8th day	3rd day	5 days	3 days

Every case of cholera whether ending fatally or not is potentially a dangerous source of the disease, but with regard to the question of a healthy 'carrier,' of course, the fatal cases are out of consideration. Of the 49 cases, who recovered, no less than 46 were free of the vibrios within seven days of the illness. Although it was very desirable to keep these cases for longer periods under observation, yet as most of them were pilgrims it was very difficult to prevent them from leaving the hospital as soon as they recovered sufficient strength to leave the bed. But no case was discharged unless the stools were negative for at least 2 days. The longest period of observation was 19 days in 2 cases only. Of the 3 exceptions who were not free of the vibrios within 7 days, one became free on the 7th day, one on the 12th day and one on the 15th day. Thus the longest period for which the vibrio persisted in a case in these series was 15 days. The period of observation after the stools became negative is shown in the table given below —

Days under observation after the stools were no longer positive	Stools examined (but found no vibrios) on	Number of cases
0 day	0 day	1
1 "	1 "	3
2 days	1 "	2
2 "	2 days	2
3 "	0 day	1
3 "	1 "	2
3 "	2 days	4

Days under observation after the stools were no longer positive	Stools examined (but found no vibrios) on	Number of cases
3 days	3 days	2
4 "	1 day	2
4 "	2 days	6
4 "	3 "	2
5 "	1 day	1
5 "	2 days	1
5 "	3	1
6 "	2 "	4
6 "	3 "	2
6 "	4 "	1
7 "	2 "	2
7 "	3 "	4
8 "	2 "	1
9 "	1 day	1
9 "	2 days	1
9 "	3 "	1
13 "	3 "	1
15 "	2 "	1
	Total	49

*The maximum period of 'carrying' the cholera vibrio is very short*

It may, therefore, be stated that 95 per cent of all cases and contacts are free of vibrios within a fortnight. In other words at the very outset the conditions are unfavourable for the production of the 'chronic carrier' state in cholera. Ninety-five per cent of cases being free of vibrios within a fortnight, they may be left out of consideration in so far as the 'carrier' problem is concerned. It is only about five cases in a hundred that are likely to pass vibrios for longer periods than 14 days. It is these cases in which we may look for the existence of the so-called 'chronic carrier' of cholera. Conseil (1912)(9) found the longest period of 'carrying' to be only 20 days. Sergeant's case (1912)(11) of a native woman 'carried' for 53 days. Jatta (1912)(12) found that healthy 'carriers' usually stopped passing the vibrios in 3 to 5 days, but 15 per cent continued to

caused 99·83 per cent and the 'carriers' 0·17 per cent of cholera cases. This is a vast difference showing that in connection with the spread of cholera, the 'carrier' may be considered for practical purposes to have no importance whatever.

It will be seen from the foregoing paragraphs what I have endeavoured to show is that there are no 'chronic carriers' in cholera. Ninety-five in a 100 cases are free of vibrios within 14 days, the rest within 1½ months, and the part the latter play in the causation of cholera is negligible. In typhoid fever cases are known to have been 'carrying' for years and numerous instances are on record of 'chronic carriers' who have given rise to epidemics after years of 'carrying'. This is far from being the case in cholera, here the cases are free of vibrios soon after the attack—within weeks, not months nor years. The chances of such cases being infectious a couple of months after recovery from the disease are very remote indeed. I am not aware of any epidemic of cholera that has been traced to a 'carrier' of two months' standing.

*Epidemiological evidence is against the existence of the 'chronic carrier' of cholera*

The limited geographical distribution of cholera is an evidence against the chronic 'carriers'. If there were 'chronic carriers' of cholera, then how is it that in spite of all the modern facilities of travel, cholera should be confined to a few endemic areas in the world and all other countries be entirely free from it. Surely typhoid fever is not so limited in its distribution throughout the world. If otherwise healthy people (for so the 'chronic carriers' are) could infect others for years, then these infective agents would have had a much wider distribution in the world and so would have been the distribution of Asiatic cholera. Macnamara (22) writes about cholera that 'after having been a certain time epidemic in a locality, it entirely disappears, unless in its endemic area. In considering this feature of the disease we must again appeal to its history. I have already pointed out the fact that cholera is hardly endemic in any country beyond the peninsula of India, the eastern provinces of the Bay of Bengal and Java. It has appeared over Europe and America on several occasions, but after exercising its baneful influence for a period of two or three years it has gradually died out and disappeared, until again rekindled by a fresh importation of the disease from India'. Again, it often happens that some of the non-endemic areas (such as those in India) are attacked by cholera one year and are entirely free from the disease for a number of years in succession after which they get a visitation again. If there really were an effective number of infectious 'carriers' in the community, one would not find a number of such free years immediately following an extensive epidemic. The free years are not due to the immunization of the community by the epidemic, because there are other areas which have had severe epidemics every year for the 50 to 60 years of available records. They are not due to unfavourable meteorological conditions either, because such conditions are not likely to be maintained for a number of full years in succession. Further, if fresh importation of the virus by patients takes place in any one of these years, an epidemic will occur in that year.



Thus in the Punjab severe epidemics have followed every one of the Kumbh Fairs of Hardwar. From these big fairs that take place every twelfth year with a concourse of about 2 million pilgrims at the chief bathing day, the Punjab is invariably infected, followed invariably by a severe epidemic. Such cholera-free years as mentioned above show the non-existence of 'carriers' and are due to the absence of importation of the disease from outside.

Those who have followed the history of Asiatic cholera have emphasized the fact over and over again that the excursions of this disease into other countries, viz., the various pandemics outside the confines of India, have been caused by patients suffering from cholera and have always followed an increased activity of the disease in its endemic areas. To quote Macnamara (23) again 'a sixth characteristic feature of epidemic cholera is that every outbreak of this disease beyond the confines of British India may be traced back to Hindustan through a continuous chain of human beings affected with the disease or through articles stained with their dejecta. Thus has the malady been propagated from one human being to another until its influence has spread from east as far as the western shores of America. But cholera has never appeared in America unless Europe has been first affected, it has never broken out in the west of Europe unless the eastern part of the continent has been previously under its influence. And here let me draw the reader's attention to the fact that Australia and other large tracts of country have as yet been free from Asiatic cholera, and that these places are separated from India by extensive oceans or seas.

We have traced Asiatic cholera on several occasions to Mauritius, but always after the arrival of vessels from India with persons on board who had been among those suffering from cholera. The same thing has occurred in America, Guadaloupe, the islands of Grand Canary group and so on. In fact I may with confidence challenge any one to cite an instance of epidemic cholera occurring beyond the precincts of British India, unless connected by a direct chain of cases with an outbreak of the disease in India. That is why the seaports of a country are the first to be affected, and that is why cholera cannot cross a desert though it may circumvent it. Many other such facts from the epidemiological behaviour of cholera may be cited to show that there are no 'chronic carriers' but the disease is propagated from place to place by patients suffering from it.

*Extended search has failed to find a 'chronic carrier' of V. cholerae*

Extended search has been made by many observers in many parts of the world to find 'carriers' in the general population but even in the endemic areas no 'carrier' of Koch's vibrio has been found. Tomb and Maitra (1921) (24) have examined a large number of stools of healthy people in the endemic area of Asansol, Bengal. They examined the whole stool, instead of a small quantity of it, by what they call the 'open bowl' method of stool examination. Other workers examined the stools of the general population in other endemic areas of Bengal. But none has succeeded in finding a 'carrier' of cholera. Thus, Tomb and Maitra (1926) (25) write it should be emphasized that in no instance

have we ever been able to find agglutinating vibrios in the stools of either survivors or of contact "carriers" three to four weeks after the cessation of an epidemic'

We endeavoured to find healthy 'carriers' in the pilgrim centre of Hardwar. Now in Hardwar cholera is often present in the hot weather, though it is not endemic, but is brought by the pilgrims and is confined in a large measure to them. From March 1927 to September 1928, we examined by the 'open bowl' method the stools of 2,898 healthy people comprising 648 pilgrims and 2,250 residents of Hardwar, but did not find a single healthy 'carrier' of Koch's vibrio. Indeed, it was not expected to discover any among the residents of a non-endemic area, although in Hardwar the residents are in close contact with the infected pilgrims. On the other hand it is a common thing to find pilgrims, coming from the endemic areas of cholera, reaching Hardwar ill with the disease. Direct search has failed to reveal the existence of 'chronic carriers' among the general population. This brings us to the consideration of another kind of 'carriers,' e.g., the 'carriers' of what are called the 'magglutinable' vibrios.

*The 'carriers' of 'magglutinable' vibrios have nothing to do with the origin and spread of cholera.*

Can the 'magglutinable' vibrios give rise to outbreaks of epidemic cholera? There is overwhelming evidence to show that the 'magglutinable' vibrios as such do not cause epidemic cholera. The utmost that can be said in their favour is that they may cause a disease simulating cholera—the so-called sporadic or mild cases. Flu (1914) (26), Castellani (1915) (27), Chalmers and Waterfield (1916) (28), Mackie and Storer (1918) (29), Jorge Ricardo (1920) (1) and others have reported cases of a cholera-like disease whose stools on culture yielded only 'magglutinable' vibrios. But these were certainly not instances of epidemic cholera. It is generally agreed that epidemic cholera is not caused by the 'magglutinable' vibrios. If they ever cause it, they can only do so when they get changed into the 'agglutinable' form. It is doubtful if such a change takes place at all. But when this change has occurred they have lost their own existence. It is not the object of the present paper to discuss the relationship between the 'agglutinable' and the 'magglutinable' vibrios. We do not propose to deal with this question but shall only refer to the suggestion put forward by some observers, namely, that the 'carriers' of 'magglutinable' vibrios constitute the reservoir of epidemic cholera. There is very little evidence in favour of this view. The fact that there are no 'chronic carriers' of the 'agglutinable' vibrio does not mean that the reservoir must be found in the 'carriers' of the 'magglutinable' vibrios. In this connection stress is laid by some writers on the observation which shows that the 'agglutinable' vibrios may lose their specific quality of agglutination. To this we have no objection, but we find no evidence in favour of the suggestion that the 'magglutinable' vibrios constitute the reservoir of epidemic cholera. If the existence of the 'chronic carrier' has not been proved, the change of the 'magglutinable' vibrio into the 'agglutinable' form is also not demonstrated. Further, even if it were possible to change the agglutinating reaction either way,

at will, we would still be far away from the solution of the problem. It has never been suggested by anybody that the 'inagglutinable' vibrios occupy the same position of importance in relation to the aetiology of cholera as does the vibrio of Koch. For those, therefore, who agree with Koch that the causative agent of cholera is the *V. cholerae*, the question is, do the 'inagglutinable' vibrios cause cholera? As mentioned above, cases of a cholera-like disease have been observed who yielded only 'inagglutinable' vibrios. But can it be really said that those cases were caused by the 'inagglutinable' vibrios found in the stools? It is possible such cases were due to some other disease in which the presence of the vibrio was a mere coincidence. But supposing they were cases of cholera, is it not possible that they were caused by an 'agglutinable' vibrio of low virulence which lost its agglutinating power on reaching the lower part of the gut or soon after leaving it? In this connection it is well to quote from the opinion of those who have cited such cholera-like cases in proof of the pathogenic power of the 'inagglutinable' vibrio in the causation of cholera. Thus, Tomb and Maitra (1926) (25) writes 'It may be of interest to state that on three occasions only have we been able to isolate agglutinating vibrios from the water of ground tanks known to have been contaminated within the previous 24 hours by epidemic cholera dejecta, the agglutinating vibrio having in all other instances changed into the non-agglutinating form before reaching the laboratory for cultivation'.

Are the 'carriers' of 'inagglutinable' vibrios infectious at all, i.e., can they cause cholera? According to Tomb and Maitra 30 per cent of the inhabitants of the endemic area of Asansol, Bengal, are 'carriers' of 'inagglutinable' vibrios. That there may be no cholera in a locality for long periods, in spite of the presence in the population of such a large number of these 'carriers,' is an admitted fact and shows the absence of their infectious power. They have no effect on immunizing the population because when the true disease comes it is as fatal as ever. To say that they become infectious only when they get changed into the 'agglutinable' form, means the negation of their infectious power and the negation of their very existence in so far as cholera is concerned. How many of these are really 'carriers'? The vibrios are commonly found in the drinking water. How many of these 'carriers' simply pass out in their stools the vibrios they have ingested in the drinking water which somehow reach the alimentary canal as does the cholera vibrio in cases of cholera?

Again, in how many in a hundred of these 'carriers' do 'inagglutinable' vibrios change into the 'agglutinable' form say in the course of one year? As the change itself has not been demonstrated to occur what can anybody possibly say with regard to this question? But supposing this change naturally did take place then the numerical probability of such a change to occur will be found, by the most liberal estimate, to be the same as the numerical probability of importation of the true vibrio of Koch by patients suffering from cholera. Thus in a million people in Asansol, there would be 300,000 'carriers' of 'inagglutinable' vibrios, if 30 per cent of the population were 'carriers'. It, as shown by actual observation, there occur annually in Asansol for every million of population 3 to 4 independent

outbreaks of cholera and of the cholera-like disease, and they be attributed to the 'carriers' of 'magglutinable' vibrios, this will mean that the change takes place in 3 to 4 'carriers' in a hundred thousand. Making, however, a liberal estimate, taking one in a thousand 'carriers' undergoing the change, we will as a consequence of this change have 300 people in a million capable of causing outbreaks of cholera. But do 300 people in a million in Asansol not visit or come from cholera-infected areas in India annually and have they not equal, if not greater, potentiality of importing the virus and starting outbreaks of the disease? This number of 300 will still further be reduced if the percentage of 'carriers' be taken less than 30. As it was found necessary by Tomb and Maitra to examine the whole stool to arrive at this figure, the number of vibrios in some must have been too small to be of any importance.

*The maximum period of 'carrying' the 'magglutinable' vibrios is also very short*

We have seen that the 'carriers' of the vibrio of Koch are, in the first place, temporary in nature and then they are too feebly infectious to be of any significance for practical purposes. With respect to the origin of cholera, the 'carriers' of 'magglutinable' vibrios are of still lesser significance. In the United Provinces we found them to be far less numerous than those found by Tomb and Maitra in Asansol. We were unable to attribute a single outbreak of cholera to them. All fresh epidemics in the United Provinces were caused by the true virus of the disease imported by cases suffering from epidemic cholera.

In the worst cholera district in the United Provinces we selected a number of villages which frequently suffered from cholera. With a laboratory on the spot, stools of inhabitants of these villages were examined by the 'open bowl' method. From September 1927 to November 1928, stools of 933 healthy people were examined and 'magglutinable' vibrios isolated from 61 of them. In other words about 6 per cent of the healthy population in the worst cholera villages in the United Provinces showed 'magglutinable' vibrios in their stools. Five of the 'carriers' were followed up for a period of about one year, the stools of one of them were examined daily and of each of the remaining four on alternate days. The result of the observation is given in the table below.

The number of these cases is very small, but the most evident feature of all was the scarcity of occasions on which the stools were positive. This intermittent manner in which the 'magglutinable' vibrios appeared in the stools was so striking as to arouse the suspicion of the occasional find being due to an error of contamination. It is unsafe to arrive at any conclusion from these five cases as to the chronic nature of the 'carriers' of 'magglutinable' vibrios. It is possible that such 'carriers' are free of the vibrios after 3 or 4 months. None of them gave a history of a previous attack of cholera, though it is possible they might have had a mild attack which they did not notice. As cholera was raging from April to July 1928 in the locality in which Shyam Lall and Pachkauria lived, it is possible they might have been instances of reinfection.

## NAME OF 'CARRIERS'

	Ram Pershad first detected on 15th Sept, 1927	Shyam Lal first detected on 13th Sept, 1927	Ramsagar first detected on 17th Sept 1927	Badri first detected on 15th Sept, 1927	Pachkauria first detected on 17th Sept, 1927
November 1927	0	6	9	2	12
December 1927	4	6	4	4	10
January 1928	0	0	2	0	1
February 1928	1	0	0	0	1
March 1928	0	0	0	0	0
April 1928	0	1	0	0	0
May 1928	0	1	0	0	0
June 1928	0	0	0	0	1
July 1928	0	0	0	0	4
August 1928	0	0	0	0	0
September 1928					0
October 1928					0
November 1928					0

The numbers are the number of days on which stools were positive

In these cases the whole stools were examined by the 'open bowl' method which is an intensive method for the isolation of vibrios. In spite of this the vibrios were isolated on strikingly few occasions. This shows that the 'carriers' of the 'inagglutinable' vibrios pass the vibrios in very small numbers. Now, even if the role of the 'inagglutinable' vibrios in the causation of cholera were the same as that of the vibrio of Koch, the very small numbers in which they are passed would reduce the importance of the 'carriers' of them to a very low level indeed.

Twenty cases who had recently recovered from an attack of cholera were followed up for one month. 'Inagglutinable' vibrios were found in the stools of four of these cases, in three cases only once and in one case only twice during the entire month of observation. The whole stools were examined by the 'open bowl' method. Here, once more, we see the striking scarcity in which the 'inagglutinable' vibrios were isolated from the stools.

Excluding the pilgrims, Hardwar is a very healthy place as far as cholera is concerned. Out of 2,501 stools of the residents of Hardwar examined, only 10 showed 'inagglutinable' vibrios. These positive cases occurred chiefly during the time of the Kumbh Fair when several million pilgrims visited Hardwar.

From the above it will be seen that the 'carriers' of the 'inagglutinable' vibrios also become free of them within a comparatively short period of time. The 'inagglutinable' vibrios may persist somewhat longer than the vibrio of Koch, but they probably die out in the course of 3 or 4 months. We removed some of these 'carriers' from their old surroundings and gave them to drink water free from vibrios. Although the number of men so examined was too small and the period of observation too short to enable one to draw any conclusion, yet it appeared as if the 'carriers' became free when removed from their homes and put on boiled water, showing vibrios again, in some cases, on return to their homes.

*The only reservoir of cholera are the patients suffering or recovering from the disease*

If there are no 'chronic carriers' of cholera, how then is the disease carried over from year to year? This is done by the actual cases of cholera who are highly infectious during the fully developed disease (about 4 days). A few may be infectious though less so during the period of convalescence for 14 days and a still lesser number may have doubtful infectious power during the incubation period for 4 to 5 days.

These are cases that may be called 'carriers' of cholera and no others, in them lies the real danger of cholera infection and they alone are responsible for the spread of the disease from place to place. A locality where such cases occur all the year round independent of importation may be called the endemic area of cholera. There can be no cholera anywhere in the absence of such cases provided none comes to it from the endemic areas. The origin and spread of cholera in India can be traced always to the presence of acute cases of cholera all the year round somewhere in India. Those areas where cholera is not endemic have nothing to fear from 'chronic carriers,' for, happily they do not exist. Such non-endemic areas which suffer severely from cholera from year to year would remain perpetually free if actual cases of cholera were not present all the year round somewhere in India.

### SUMMARY

It seems to be the general belief that there are exactly the same kind of 'carriers' of cholera as there are of typhoid fever. In typhoid fever the 'chronic carrier' state is held to be due to the infection of the gall bladder. Some workers have sought to prove that the infection of this viscus is responsible for the production of the 'chronic carrier' state in cholera also. It has been pointed out in this paper that there are no 'chronic carriers' in cholera, that there is no similarity between the two diseases in this respect. There is no chronic infection of the gall bladder by *V. cholerae*. The fact that the *V. cholerae* has been found in the bile of a case who had died of cholera does not prove that the condition was a chronic one.

The majority of cholera cases get free of the *V cholera* within a few days. Our results of observations in Hardwar agree with similar results of other workers elsewhere. Ninety-five per cent of all cases and contacts of cholera are free of the vibrios within 14 days, the remaining within 1½ months. There is, therefore, no possibility of any of them becoming a 'chronic carrier'.

It is very doubtful if the few exceptional cases, that may 'carry' for the longest period of 1½ months, are infectious at all. It must be clearly understood that the presence of the *V cholera* in the stools of 'carriers' does not always mean that they are infectious. In other words, such cases may be 'carriers' of the vibrio for 1½ months, but they may not be 'carriers' of the disease for the same period. The *V cholera* being a delicate organism has very little chance to cause cholera unless passed in much larger numbers than those passed in the stools of 'carriers'. The real thing will be to trace definite outbreaks of cholera to these 'carriers'. I am, however, not aware of any outbreak of epidemic cholera that has been traced to a 'carrier' of two months' standing.

Epidemiological evidence disproves the existence of the 'chronic carriers' of cholera. The limited geographical distribution of cholera is evidence against such 'carriers'. The peculiar connection of cholera with fairs and pilgrimages, the mode of spread of the disease from India to Europe and America and its inability to cross oceans and deserts are all in evidence against 'chronic carriers'. The history of cholera is full of events to show that every outbreak of this disease beyond its endemic areas has travelled stage by stage through a continuous chain of human beings affected with the disease.

Extended search has failed to find a 'chronic carrier' of *V cholera*. We examined the stools of several thousand people in the worst cholera areas in the United Provinces of India but did not find a single 'carrier' of Koch's vibrio. Vibrios that did not agglutinate with the serum of *V cholera* were found in the stools of about 6 per cent of healthy people. We found no evidence to show that the 'carriers' of the 'inagglutinable' vibrios had anything to do with the origin and spread of cholera.

The reservoir of cholera is not the 'chronic carriers' of *V cholera*, because they do not exist, it is also not in the 'carriers' of the 'inagglutinable' vibrio because they do not cause epidemic cholera. The real reservoir is in the presence in the endemic areas of patients suffering or recovering from cholera. The only source of the infection of epidemic cholera are patients suffering from the disease, in the acute stage for about 4 days, also some though to a much lesser extent in the convalescing stage for about 14 days, and perhaps a few in the incubation period for a few days.

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have alluded to this question and have very minutely described the mode of spread of a number of epidemics from the endemic areas on what Bryden calls the 'epidemic highways' Sir Leonard Rogers has recently referred to it, but from the time of Bryden to the present day opinions about this question have been based entirely on the study of available mortality records. It is hardly necessary to point out that in so far as the definition of an endemic area is concerned, a study of the mortality records alone may lead to entirely erroneous conclusions. Thus from the mortality records the fact, that a revenue division of a province was never free from cholera for a single year out of the last 30 years, was taken by Sir Leonard Rogers to be the chief reason to say that cholera was endemic in that division. But it is not quite correct to state that by endemicity is meant the mere *presence* of cholera from year to year. It is quite possible for cholera to be present in an area from year to year for a long series of years and be entirely due to importation. As an example of this may be cited the pilgrim centre of Hardwar. The disease is so regularly brought by the constant flow of pilgrims that many people consider Hardwar as an important endemic area of cholera. They are surprised to hear that Hardwar is not an endemic centre of cholera at all.

It must, therefore, be clearly stated what we mean by an endemic area of cholera. It is generally agreed that an endemic area means an area in which the disease is present over a long series of years *independent of importation*. It will, therefore, involve not an inconsiderable amount of labour to map out the endemic areas of cholera in India but it will certainly be worth while to do so from the prevention point of view. It will be necessary for this to make inquiries and observations on the spot continuously for a number of years before forming any definite opinion because otherwise one is likely to arrive at wrong conclusions. As an instance may be mentioned again the statement of Sir Leonard Rogers, who thinks some of the Sub-Himalayan divisions of the United Provinces to be endemic areas of cholera, on the supposition that they have never been free from cholera for a single year for the last 30 years. But as a matter of fact these divisions are more liable to be invaded by cholera than others on account of the proximity of Nepal, because Nepal is an important source of cholera. There is no reference in Sir Leonard Rogers' paper to this important source, because Nepal, which is a kind of an autonomous state, does not publish mortality figures. An opinion about this question, therefore, which is based on the study of published mortality records alone is open to serious objections. Lieut-Col C L Dunn, C I L, D P H, I N S, Director of Public Health, United Provinces, corroborates this statement about Nepal, from his experience of 17 years' work in the United Provinces. He thinks cholera is imported into Nepal from Bengal and then spreads to the United Provinces.

We still do not know a great deal about the epidemiology of cholera, but there are a number of facts known to have been confirmed by numerous observations. From these facts it may be stated, not without reason, that the endemic foci of cholera must be comparatively few, well defined, clear cut and limited.

areas There can hardly be any doubt about the fact that temperature does influence the prevalence of cholera Cholera is not found in cold countries It has reached such countries but has never been able to become established in them for more than two or three years Also in some parts of India where there is cold weather the disease subsides or entirely disappears during the cold weather The critical temperature appears to be  $60^{\circ}\text{F}$ , and the temperature of the previous month also has its influence Thus everywhere in India where the winter temperature falls below  $60^{\circ}\text{F}$  for two months in succession, cholera at the same time either disappears or falls to a very low level Such an area is unlikely to be an endemic focus of cholera

One can understand how the disease is kept up in non-endemic areas It is brought from outside, breaks out in an epidemic form, and dies out in the cold weather If, therefore, in such localities a fresh epidemic breaks out, the disease must have been imported again But how the disease is kept up perpetually in the endemic areas is not so easy to understand As has been stated before we do not know much about the reservoir of cholera It is a point of considerable epidemiological importance, to be able to have definite information about the reservoir of the disease It is from this point of view that I propose to take this opportunity in order to draw attention to certain observations that were made in the course of an enquiry in the United Provinces

It is generally accepted by the medical profession that the essential cause of cholera infection is the classical vibrio discovered and originally described by Robert Koch This vibrio is constantly associated with cases of Asiatic cholera, nobody has any doubt about it The copious stools of cholera patients contain the organism in almost pure culture and it is one of the easiest of procedures to isolate it from such stools It is striking to note the abundance in which the organism is present in the stools of the patient during the first few days of the disease and it is equally striking to observe that after the first few days it is no longer to be found in the majority of cases This brings us to the consideration of the 'carrier' problem of cholera Koch did not give us the solution of this problem and in subsequent work on it, unfortunately the analogy of typhoid fever was carried too far Greig has written a good deal on this subject He had the work on typhoid fever before him and he endeavoured to show that there were exactly the same kind of 'carriers' of cholera as there were of typhoid fever This view was readily accepted, it soon found place in the textbooks, and the general practitioner now has no doubt that the 'carrier' question of cholera is a settled fact But this phase of Greig's work is still to be confirmed Stools of a large number of people have since been examined in the endemic areas of cholera in India as well as abroad and yet a 'carrier' of cholera has not been found As has been said before, the majority of cholera patients soon become free from the vibrios, a few may pass it during the convalescence, but it appears as if all become free after a comparatively short period of time We have examined the stools of several thousands of healthy people in the worst cholera areas in the United Provinces and we are of the opinion that the Koch's vibrio

is passed in the stools during the incubation period, during the disease, and during the convalescence only, and not afterwards. Chronic carriers of typhoid fever undoubtedly exist but in the case of cholera the existence of chronic carriers has not been proved. It is not logical to expect to find identical conditions with respect to carriers of cholera and typhoid fever because epidemiologically the two diseases behave differently. The limited geographical distribution of cholera is in itself a strong presumption against the existence of chronic carriers. We know that it is doubtful for cholera to reach countries such as Australia which are isolated by long voyages from the endemic areas. It has never appeared in America unless Europe has been first affected, and it is unable to cross a desert of any dimensions. If these epidemiological facts can be taken to mean anything, they can also be adduced as evidence against the existence of chronic carriers. It is, therefore, not without reason to state that there are no chronic carriers of Koch's vibrio—at least none has so far been discovered. Not only are there no chronic carriers of the cholera vibrio, but the vibrio has never been found in the drinking-water, etc., without cases of cholera being present in the neighbourhood. The vibrio may be found in the drinking-water only when a fresh supply from cases of cholera is available. In other words it is unable to live a saprophytic existence.

Such is the behaviour of the vibrio of Asiatic cholera. It is an agglutinable vibrio, that is to say, the vibrio met with in all the epidemics of cholera agglutinates with the anti-serum of the standard Koch's vibrio. This vibrio has never been found in the stools of healthy people nor in the drinking-water at a time when there is no cholera prevailing. But other vibrios are commonly found both in the stools of healthy people and in the drinking-water throughout the year, even when there is no cholera about. These vibrios in other respects are hardly distinguishable from the vibrio of Asiatic cholera but they do not agglutinate with its anti-serum. The suggestion has, therefore, been put forward by some workers that these inagglutinable vibrios constitute the reservoir of cholera. It has to be assumed, however, that before they can take on the property of causing outbreaks of epidemic cholera, it is necessary for them first to change into the agglutinable form. This change has not been demonstrated to occur, still less is known about the conditions which bring about this supposed mutation in nature.

As has already been stated healthy people passing inagglutinable vibrios are commonly encountered. I was informed by one of the delegates to the interchange of Health Officers who visited India this year, that in the Philippines all carriers of inagglutinable vibrios are considered for practical purposes as true carriers of Asiatic cholera. The Indian Research Fund Association instituted an inquiry about this question which was carried out in the United Provinces. It was proposed to investigate the origin of cholera in the United Provinces and its possible connection with the carriers of the inagglutinable vibrios.

Mortality records for the last 60 years are available for these Provinces. From a study of these records two epidemiological phenomena came into particular prominence. They promised to afford very suitable fields for investigation on the proposed lines. It was found that some of the district suffered much

more from cholera while others were comparatively free. It was found that the disease reached its highest incidence during the months of May and June, decreased with the advent of the monsoon, and subsiding with the approach of cold weather entirely disappeared from the whole of the United Provinces during the months of January and February. Taken individually, however, all the districts even the worst cholera ones enjoyed complete freedom from cholera for the greater part of the year, and the whole of the United Provinces comprising 48 districts remained absolutely free from the disease during the months of January and February. This has been confirmed by investigation on the spot.

We were interested to know how the disease broke out afresh after it had entirely disappeared during the cold weather months. It was, therefore, necessary to investigate every fresh outbreak in the whole of the United Provinces. This required an organization on a large scale and a number of workers beyond our means. But thanks to the excellent health service in the United Provinces with the help given by the district medical officers of health, we were able to investigate every one of these outbreaks.

We also selected an observation area situated in the worst cholera district. This area consisted of a number of villages known to be liable to frequent outbreaks of cholera. Maps were prepared for these villages showing wells, tanks, houses, etc. A census was made of the population and each individual was entered in the register for follow up work. They were divided into four groups, viz., survivors of the cases of the last epidemic, contacts of these cases, the rest of the population and the new-comers. A laboratory was established on the spot. Stools of all these people and also the drinking-water were systematically examined for the presence of vibrios. Every outbreak of cholera in this area was investigated.

Inagglutinable vibrios could be found in the drinking-water and also in the stools of the healthy people in these villages. About 6 per cent of the population showed inagglutinable vibrios in their stools. We have failed to discover any relationship between these vibrios and the origin of cholera. Not a single outbreak could be traced to a carrier of the inagglutinable vibrios. All outbreaks were found to be due to importation. This brings us again to the question of the reservoir of cholera. Take the case of a district which is free from cholera for a year or two and gets a visitation every second or third year. Take, for instance, the case of the United Provinces. This area remains free from cholera for at least two months in a year. It has a cholera season, that is to say, the disease breaks out in a virulent form every year during the months of May and June. From what source then does the infection take its origin in such areas? Does it arise from chronic carriers? This, as we have seen, is not borne out by the observed facts. The existence of chronic carriers is not proved. Does it then arise from the inagglutinable vibrios that may be found there? Our work in the United Provinces shows that this does not take place, nor have the inagglutinable vibrios been proved to cause epidemics of cholera in any other area. We, therefore, think that the reservoir of cholera must be the actual cases of cholera in the

active stage of the disease, in the incubation period of a week or so, and in the convalescent period of about 15 days. The disease arises from such cases who import the virus into non-endemic areas. We may refer to such cases as 'acute' carriers. The truth of this statement has stood the test of numerous observations ever since the history of the disease began. But research work on cholera has been too much of the laboratory kind, and the investigation of individual cases with a view to tracing the source of infection has not received the attention due to it. If proof of this conception of the reservoir of cholera is required it has been furnished with corroboration by the laboratory, at least in so far as the United Provinces are concerned. I consider it essential to draw particular attention to it. Let this hypothesis of the 'acute' carrier to be the only or at least the chief reservoir of cholera, instead of either the chronic carrier or the magglutinable vibrio, be made the subject of further inquiry to prove or disprove our results, for if any work on cholera is to be of any practical value we must entertain no doubts on this question. We started to work with the idea that the origin of cholera possibly takes place either from chronic carriers of the true cholera vibrios or from the magglutinable vibrios. But the results of our work did not support this hypothesis and led us to the opinion that epidemic cholera arises only from cases of cholera (either ill or convalescing or in the incubation period) and from nothing else. The real danger, therefore, lies in these 'acute' carriers. The vicious circle is kept up in the following manner — Suppose epidemic cholera is present in district A in India. An 'acute' carrier, whether a resident or a visitor, carries the disease from A to another district B and starts an epidemic there, from B another 'acute' carrier carries it to another district C and so on. When the disease is raging at B it is possible A has become free, by that time and so on, or A may never become free, it may be a place having cases of cholera all the year round, in other words it may be an endemic area of cholera. The origin of fresh outbreaks of cholera anywhere in India, therefore, depends on the presence of actual cases all the time somewhere in India. It is immaterial where this place is provided free communication takes place with it through human beings. If actual cases of cholera are not present, all the time anywhere in India, this hypothesis falls to the ground. The above condition fulfilled, the disease is carried from place to place by the movements of the actual cases either ill, convalescing or in the incubation period, that is to say, the 'acute' carriers. Any concourse of human beings of such a nature and magnitude as to ensure the presence of an 'acute' carrier, is liable to an outbreak of cholera especially when the temperature of the season is favourable.

# A FEW OBSERVATIONS ON MYCETOMA    A PRELIMINARY COMMUNICATION

BY

A VASUDEVAN, M B & B S,  
*Curator, Pathology Museum,*

AND

N SESHADRINATHAN, M B & B S,  
*Department of Bacteriology,  
Medical College, Madras*

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THE term 'Mycetoma' is used to denote chronic inflammatory lesions with swelling and sinuses through which granules composed of fungal elements are discharged periodically. This condition is most common in the foot though it occurs elsewhere and is commonly called the 'Madura foot'. It is caused by a great variety of fungi occupying a wide range in the mycological scale and widely differing from one another. For example one variety, the black mycetoma (African variety), is caused by *Aspergillus bouffardi* which is an Ascomyces while another, a white variety (Vincet's type), is caused by *Nocardia indica*, which is an imperfect fungus. The structural and cultural characters of these two fungi are widely dissimilar. The different fungi that cause mycetoma are classified under two broad divisions (a) the *maduromyces*, and (b) the *actinomyces* and the lesions produced by them are called maduromycosis and actinomycosis respectively. The maduromycosis are those varieties in which there are present grains composed of large segmented mycelial filaments and possessing well defined walls and usually clamydospores and actinomycosis are those varieties in which there are present grains composed of very fine non-segmented mycelial filaments in which the walls are not clearly defined from the contents and clamydospores are absent. In each variety are numerous fungi and a detailed description of them is beyond the scope of the present article. It is thus evident that the term mycetoma is not specific but used in a loose sense to denote a clinical condition.



The object of our present investigation is primarily to arrive at the exact mode of infection. It is assumed that the fungus gains entrance through wounds and abrasions, as for example thorn pricks in the feet. In South India, except the few well-to-do classes, the majority of people go about with bare feet and it is in such classes of people, e.g., the ryot, that mycetoma is common. In many cases there is a definite history of a thorn prick or injury with splinter of wood and such objects have been removed from mycetomatous lesions years after the injury. It is probably for this reason that the condition is most common in the feet. One of the commonest thorns in India is the ubiquitous prickly pear. Then there are the bamboo and the acacia also widely distributed. It is possible that the fungus whether a *maduromyces* or an *actinomyces* resides saprophytically on such thorns and is carried by them into the feet. Or again, the fungus as a saprophite in soil (varieties of *actinomyces* are known to exist in the soil) may get in subsequently through wounds caused by thorns not themselves contaminated. It is necessary, therefore, to search for these fungi in thorn-bearing trees and plants, e.g., the bamboo, prickly pear, acacia and also the soil. But in nature a variety of other fungi are usually seen on such objects, and it would be almost impossible to investigate all of them with the possibility of their being the fungus in question. But if we have the appearances of the usual fungi causing mycetoma, as grown on such natural objects, it would facilitate the works considerably. With this view, successful attempts were made to grow them on tender bamboo twigs, the succulent stem of the common prickly pear and the soil.

The three varieties of fungi, the white, red and black were used for these cultures —

The following cultures were made —

(1) A variety of red mycetoma grown on tender bamboo twigs. A small scarification was made by a knife on the skin of a tender twig of a bamboo which had been autoclaved and a small quantity of crushed material from a growth of red mycetoma was rubbed into it. The twig was made to stand vertically in a test tube at the bottom of which was kept a little water and the tube was sealed with paraffin and incubated at 37°C. In from about two to two and a half months growth was appreciable and the culture continued to grow for some months and then stopped, apparently after all the food material from the twig had been used up. The growth obtained had the following characteristics. It was dark reddish brown, dry, granular and heaped up at the base not unlike a miniature red ant-hill.

(2) A variety of white mycetoma (actinomycotic) grown on the common prickly pear (*Opuntia vulgaris*)

A piece of the succulent stem of the common prickly pear was autoclaved and a little of the crushed material from a white variety of mycetoma was implanted on to it over a small area of scarification. A few drops of sterile water were kept in the bottom of the test tube which was sealed with paraffin and incubated at 37°C. Growth was appreciable about one and a half months later and the culture continued to grow for some months. The growth had the following

characters It was yellowish white, rounded, raised, mammillated and moist It was soft, tough, elastic and not easily crushed and was firmly adherent to the prickly pear into which it had evidently grown and from which it was difficult to separate while crushing it for microscopical examination The growth on the prickly pear was more luxuriant than on the bamboo

(3) White and black varieties grown on 'Soil Medium'

Since it is difficult to handle and observe ordinary soil for purposes of culturing fungi, we used a 'soil extract' medium This consists of an aqueous extract of finely pulverised earth strained through fine muslin to which potassium phosphate is added and the extract incorporated with plain agar and autoclaved For all practical purposes, it may be said to be equivalent to the ordinary soil for purposes of culture as no extraneous nourishment such as the usual meat extractives, etc., was added to it and the fungus could utilize only the nourishment present in the soil A specimen of white mycetoma and one of black mycetoma were grown on this medium The growths had the following characters —

(a) White variety (Actinomycotic)

The growth is slow but at the end of two months a definite growth was visible It is whitish, granular, raised and has a 'heaped up salt' appearance It does not seem to have penetrated into the medium nor has it spread superficially

(b) Black variety

The main mass of the growth is pale whitish in colour, slightly raised and granular and in the centre (oldest portions) it is black in colour which colour seems to increase both in intensity and extent as the culture ages All round there is a pale whitish filmy halo which is seen to extend into the depth of the medium

These two cultures are still growing

From the foregoing it will be seen that these fungi grow with comparative ease on pieces of bamboo, prickly pear, soil and perhaps in a variety of other plants evidently as a saprophyte This would also suggest a very wide distribution in nature It is said that mycetoma is common in districts with black cotton soil The truth of this statement is difficult to estimate as statistics of mycetoma are imperfect and so far as I am aware no authentic, regular mappings of this disease exists This is an important field of investigation It would be interesting to find out how these fungi would grow in the different kinds of soil, e.g., the alluvial, black-cotton soil, sandy, red, highly nitrogenous boggy soils, etc Would the kind of soil most favourable for such experimental growth correspond to the district most commonly infected? This would give us an idea of the endemicity of this disease Attempts at investigation in this direction are being made

There is another method of attacking this problem That is, to search for growths of the fungi in natural conditions such as the bamboo, prickly pear, acacia, etc., and ascertain if they correspond to the known varieties Then they could be grown on the usual favourable media and their cultural and structural

characters compared and confirmed This, of course, is a very patient and difficult task and a close co-operation of the medical, the agricultural and the forest departments is necessary for its success

This paper is intended as a preliminary communication, with the hope of encouraging work along these lines We propose to publish at a later date more details of what has been done by us and also the results of experiments being now carried on

In conclusion, we must acknowledge a debt of gratitude to Lt-Col E W C Bradfield, OBE, CIE, IMS, Superintendent, General Hospital, Madras, not only for the supply of clinical materials, but also for suggestions as to the mode of investigation and for the constant encouragement that he has kindly given us but for which this work would not have been undertaken Our thanks are also due to Mr R D Anstead, Director of Agriculture, Madras, and to Mr S Sundararaman, MA, Mycologist of the Agricultural College, Coimbatore, for their assistance

# THE RELATIVE MALARIAL INFECTIVITY OF SOME SPECIES OF ANOPHELINES IN CACHAR (ASSAM)

BY

C STRICKLAND, M D,

*Professor of Medical Entomology, School of Tropical Medicine  
and Hygiene, Calcutta*

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ALTHOUGH the main conclusion reached by myself and collaborators (1928), in our Report on Malaria in the Assam Tea Gardens, was that *funestus* and its most productive breeding-places, the perennially-flowing grassy-edged streamlets of the country, were the greatest source of the trouble, this conclusion was based, for lack of time and staff, almost exclusively on an analysis of the distribution of the malaria endemicity and the Anopheline breeding-places, very few observations having been made on the relative prevalence of the adult Anophelines in the habitations of those affected, and none at all on the mosquitoes' relative infectivity

The seriousness of these omissions being realized successful steps were taken to remedy the deficiency and the work involved is the subject of the following paper. It was the outcome of a grant from the Indian Research Fund Association to which I am accordingly indebted, and the observations being made on a group of tea estates in Cachar in southern Assam I am much obliged to my helpers there

Because when arranging the research it was felt that if an enormous number of Anophelines caught at random in the human habitations were not dissected and examined for malaria infection, one would probably not obtain a true representation of their relative infectivity, a great effort was made to obviate such a sequel. An organization was set for the purpose of catching as many of the adult insects as possible—mainly by trapping in the cooly-lines—and five laboratory assistants, each armed with a dissecting microscope and specially trained for the work, were entertained to identify and dissect the catch, one of the men being detailed to conduct the preliminary examination and preservation of the infected specimens

The scheme was designed for a year but was brought to a conclusion after 10,000 mosquitoes had been dissected in about 8 months (April to December 1927)

The following table shows the result *per species* —

TABLE I

No	Name of species	Number dissected	Gut infected	Glands infected	Total
1	<i>A funestus</i>	1 489	39	19	58
2	<i>A ramsayi</i> vel <i>pseudojamesi</i>	256		1	1
3	<i>A kochi</i>	535	1		1
4	<i>A vagus</i>	1,341	1		1
5	<i>A karwari</i>	1 697	1		1
6	<i>A maculatus</i>	147			
7	<i>A jeyporiensis</i>	125			
8	<i>A culicifacies</i>	1			
9	<i>A tessellatus</i>	6			
10	<i>A acontus</i>	136			
11	<i>A fuliginosus</i>	51			
12	<i>A philippinensis</i>	2 410			
13	<i>A hyrcanus</i>	1 757			
14	<i>A jamesi</i>	8			
15	<i>A barbirostris</i>	2			
16	<i>A aitkeni</i>	3			
17	<i>A leucosphyrus</i>	36			
	TOTAL	10 000	42	20	62

It will be seen that about six of every thousand adult *Anophelines* caught in the human habitations were found infected and about two of these were at the stage infective to man

The differences in the infective-rates of *funestus*, *vagus*, *karwari*, and *philippinensis* as seen in the Table, could not, it is presumed, have been due entirely to any insufficiency of the data, the variation being too great to be accountable for solely by chance, indeed with regard to the rate shown for *funestus* there is some evidence on the point

Table II gives the *funestus* infection-rate month by month, as also does Table III, calculated for bi-monthly periods to lessen the chance of random error

TABLE II.  
*A. funestus*

Locality	Months	Number dissected	Gut infected	Glands infected	Total
Cachar	April	8			
	May	52			
	June	235	7	4	11
	July	266	5	2	7
	August	120	4	2	6
	September	71	3		3
	October	219	5	1	6
	November	382	12	9	21
	TOTAL	1,353	36	18	54

TABLE III

Locality	Months	Number dissected	Total infected	Per cent infected (about)
Cachar	April and May	60		
	June and July	501	18	3.6
	August and September	191	9	4.5
	October and November	601	27	4.7
	TOTAL	1,353	54	4.0

There is not, if one omits the first period in which so few specimens were examined, a great variation in the figures and what there is may have been due to seasonal influences, rather than to the misrepresentation of an insufficiency of data of a hypothetically constant rate. If the data had been insufficient the figures would probably have been much more variable. One may conclude then, from this periodic analysis, that probably the total number of *funestus* examined over

the whole period must have been adequate to give one a true picture of the natural infectivity of this species—under the local conditions obtaining—and its extraordinary high rate here of 4 per cent must be noted

The following records taken from Covell (1927) of the natural infectivity of *funestus* in other countries are not dissimilar from that given in Table I, and do not exhibit great variations, dependent though the figures were on the endemicity in the different localities examined

TABLE IV

Synonyms	Observer	Number dissected	Number infected.	Per cent infected
' <i>funestus</i> '	Lamborn	430		27
' <i>histoni</i> '	Perry	229		18
"	Chalam	315		38
' <i>minimus</i> '	Stephens and Christophers	64	4	
"	Lalor	81	1	
"	Iyengar	25	1	
	TOTAL	170	6	3.5

The evidence then regarding *funestus* is such as to lead us to suppose that the infective-rates not only of it, but also of *vagus*, *karwan* and *philippinensis* as shown in Table I, give a true representation of their infectivity in nature—under the conditions examined

The negative infective-rate of *philippinensis* based on the dissection of 2,410 specimens must now be noted, for Sur in Bengal has recently (1928) reported finding 3.1 per cent out of 223 specimens naturally infected. It is difficult to suggest an explanation of the discrepancy between his results and that shown in Table I. His observations were carried on during October and November, those of this survey during April to November,\* the climate of Cachar being very similar to Bengal at corresponding periods, and while his were made in villages in Krishnagar District where the spleen-indices were over 90 per cent, yet ours were from estates where in 1923 I had found the spleen-index of 373 children to be about 72 per cent. This very important matter needs elucidation.

Insufficiency of data might perhaps have accounted for such reputed carrier-species as *maculatus*, *peyponensis*, *culicifacies* and *acomtus* not showing any infective-rate, and to this extent the scheme of the experiment failed we should have been able to show reliable infective-indices for all the local species, though of course we were from the first limited to the numbers we could catch

\* We dissected 1,091 during those two months without result.

The record of infection of *ramsayi* vel *pseudojamesi* is interesting though the paucity of the numbers dissected (243) place it in the category of the species whose infective indices as shown must be doubtful. In a report on a mosquito-survey of the Bengal districts (Strickland and Chowdhury, 1927) it was stated (p 382) 'in view of the difficulty experienced by epidemiologists in the past in allocating the malaria of the Province to any specific cause, this find of *pseudojamesi* may prove of great importance'. In this Cachar survey its number and infectivity indicate that it may be about 340 times less infective than *funestus*.

The monthly tables of the dissections are appended. That for *funestus* was analysed above and pointed to a slight increase of infectivity during the rainy months (the epidemic season).

### Conclusion

It is concluded that the dissection of 1,489 naturally-caught specimens of a species is sufficient to afford one a fairly true representation of its infectivity and that Table V, judging from the numbers found and the rate at which they were infected, approximately represents under the local conditions the relative infectivity of the species.

TABLE V

Number	Species	Number found	Number infected	Infectivity coefficient <i>karwari</i> being 1
1	<i>funestus</i>	1 489	58	64
2	<i>vagus</i>	1,341	1	125
3	<i>karwari</i>	1,697	1	1
4	<i>philippinensis</i>	2,410	0	nil
5	<i>hyrcanus</i>	1 757	0	nil

Therefore without apparently having been able to establish the infectivity of many important species, the experiment has definitely confirmed the enormous relative importance of *funestus* in the malaria-problem of the Assam tea gardens, a potentiality for danger derived from two factors, its preponderant infectivity and its relatively great prevalence, and this finding has justified the stress laid in our Report on Malaria in Assam Tea Gardens (*op cit*) on the urgency of making a dead set at this species.

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## APPENDIX

*Anopheline mosquitoes dissected during the month of April 1927*

Locality	Name of species	Number dissected	Guts infected	Glands infected	Total infected
Cachar	<i>A. minimus</i>	8			
	<i>A. kachi</i>	93			
	<i>A. vagus</i>	29			
	<i>A. karwari</i>	30			
	<i>A. maculatus</i>	4			
	<i>A. jeypariensis</i>	1			
	<i>A. tessellatus</i>	1			
	<i>A. acanthus</i>	11			
	<i>A. fuliginosus</i>	7			
	<i>A. philippinensis</i>	21			
	<i>A. hyrcanus</i>	94			
	<i>A. leucosphyrus</i>	1			
	TOTAL	300			1

May 1927

Locality	Name of species	Number dissected	Guts infected	Glands infected	Total infected
Cachar	<i>A. minimus</i>	52			
	<i>A. kachi</i>	91			
	<i>A. vagus</i>	53			
	<i>A. karwari</i>	106			
	<i>A. maculatus</i>	14			
	<i>A. jeypariensis</i>	10			
	<i>A. culicifacies</i>	1			
	<i>A. tessellatus</i>	3			
	<i>A. acanthus</i>	24			
	<i>A. fuliginosus</i>	12			
	<i>A. philippinensis</i>	63			
	<i>A. hyrcanus</i>	251			
	<i>A. jamaicus</i>	1			
	TOTAL	681			

*Anopheline mosquitoes dissected during the month of June 1927*

Locality	Name of species	Number dissected	Guts infected	Glands infected	Total infected
Cachar	<i>A. minimus</i>	235	7	4	11
	<i>A. ramsayi</i>	27	..	1	1
	<i>A. kochi</i>	19			..
	<i>A. vagus</i>	23			
	<i>A. karwari</i>	142			.
	<i>A. maculatus</i>	20	..		.
	<i>A. jeyporiensis</i>	8			.
	<i>A. acontus</i>	8			
	<i>A. fuliginosus</i>	10			.
	<i>A. philippinensis</i>	115			.
	<i>A. hyrcanus</i>	112			.
	<i>A. barbirostris</i>	1	.		.
	TOTAL	720	7	5	12

## July 1927

Locality	Name of species	Number dissected	Guts infected	Glands infected	Total infected
Cachar	<i>A. minimus</i>	266	5	2	7
	<i>A. ramsayi</i>	35			
	<i>A. kochi</i>	14	1		1
	<i>A. vagus</i>	31	1	.	1
	<i>A. karwari</i>	125	1		1
	<i>A. maculatus</i>	16	.		.
	<i>A. jeyporiensis</i>	15			
	<i>A. acontus</i>	1		.	.
	<i>A. fuliginosus</i>	8	.	..	
	<i>A. philippinensis</i>	161	.	.	
	<i>A. hyrcanus</i>	88	.		
	<i>A. jamesi</i> ..	1	.	..	.
	<i>A. atkenni</i> ..	1	..	..	
	<i>A. tessellatus</i>	13		.	.
	TOTAL	775	8	2	10

*Anopheline mosquitoes dissected during the month of August 1927*

Locality	Name of species	Number dissected.	Guts infected	Glands infected	Total infected
Cachar	<i>A. minimus</i>	120	4	2	6
	<i>A. ramsayi</i>	19			
	<i>A. kochi</i>	35			
	<i>A. vagus</i>	33			
	<i>A. karwari</i>	268			
	<i>A. maculatus</i>	15			
	<i>A. jeyporiensis</i>	23			
	<i>A. aconitus</i>	2			
	<i>A. fuliginosus</i>	5			
	<i>A. philippinensis</i>	327			
	<i>A. hyrcanus</i>	186			
	<i>A. leucosphyrus</i>	1			
	TOTAL	1,034	4	2	6

*September 1927*

Locality	Name of species	Number dissected.	Guts infected	Glands infected	Total infected
Cachar	<i>A. minimus</i>	71	3		3
	<i>A. ramsayi</i>	68			
	<i>A. kochi</i>	24			
	<i>A. vagus</i>	126			
	<i>A. karwari</i>	220			
	<i>A. maculatus</i>	12			
	<i>A. jeyporiensis</i>	14			
	<i>A. aconitus</i>	1			
	<i>A. philippinensis</i>	327			
	<i>A. hyrcanus</i>	200			
	<i>A. ankeni</i>	2			
	<i>A. leucosphyrus</i>	5			
	TOTAL	1070	3		3

*Anopheline mosquitoes dissected during the month of October 1927*

Locality	Name of species	Number dissected	Guts infected	Glands infected	Total infected
Cachar	<i>A. minimus</i>	219	5	1	6
	<i>A. ramsayi</i>	65			
	<i>A. kochi</i>	66			
	<i>A. vagus</i>	412			
	<i>A. karwari</i>	230			
	<i>A. maculatus</i>	4			
	<i>A. jeyporiensis</i>	16			
	<i>A. tessellatus</i>	1			
	<i>A. aconitus</i>	3			
	<i>A. philippinensis</i>	488			
	<i>A. hyrcanus</i>	430			
	<i>A. jamesi</i>	2			
	<i>A. barbuostrius</i>	1			
	<i>A. leucosphyrus</i>	15			
	TOTAL	1,952	5	1	6

*November 1927*

Locality	Name of species	Number dissected	Guts infected	Glands infected	Total infected
Cachar	<i>A. minimus</i>	382	12	9	21
	<i>A. ramsayi</i>	29			
	<i>A. kochi</i>	131			
	<i>A. vagus</i>	491			
	<i>A. karwari</i>	352			
	<i>A. maculatus</i>	21			
	<i>A. jeyporiensis</i>	22			
	<i>A. tessellatus</i>	1			
	<i>A. aconitus</i>	63			
	<i>A. fuliginosus</i>	8			
	<i>A. philippinensis</i>	603			
	<i>A. hyrcanus</i>	257			
	<i>A. jamesi</i>	3			
	<i>A. leucosphyrus</i>	1			
	TOTAL	2,364	12	9	21

# DYSENTERY PROPHYLAXIS BY ORAL BILIVACCIN AT POONA AND SECUNDERABAD

BY

MAJOR W WALKER, M C, R A M C,

AND

CAPTAIN R C WATS, I M S

[Received for publication, February 21, 1929]

THE exhaustive investigations by Major Manifold(1 and 2) into the prevalent types of dysentery encountered in Poona revealed the fact that the great majority were bacillary in origin. He further demonstrated that *B flexner* was the most frequent infecting organism encountered.

Funds having been obtained from the Indian Research Fund Association, the task of preparing an efficient prophylactic Flexner vaccine was undertaken by the senior writer. Two strains of *B flexner*, previously isolated by Major Manifold, were used for this purpose on account of their high antigenetic properties. A vaccine containing a 1,000 million of each strain per c.c. was prepared and preliminary animal tests having proved satisfactory as regards the production of agglutinins against the five standard strains of *B flexner*, it was decided to test the vaccine on the laboratory staff. A serological examination of each individual, before the administration of the vaccine, revealed the presence of agglutinins against *B flexner* V, W, X, Z and Y in all cases. The vaccine was administered in two doses, first dose  $\frac{1}{2}$  c.c. followed by 1 c.c. after an interval of ten days. Serological tests were carried out on each individual at frequent intervals and it was found that the existing agglutinins rapidly disappeared in all cases. Further, two individuals developed acute bacillary dysentery within twenty-one days of the second dose of the vaccine. It was decided not to proceed further with human tests until more exhaustive animal tests could be carried out.

Just when matters in this line of investigation had come to a standstill, a liberal supply of Oral Bilivaccin was put at our disposal by the Director of Medical Services in India with the request that its efficacy be tested as a prophylactic against bacillary dysentery amongst the troops in the district. This definitely

determined our future line of investigation in local dysentery prophylaxis. The Bihvaccin was tried out in Poona and Secunderabad and the results are recorded herewith.

### *Preliminary Notes*

(1) It was decided to limit this investigation to British troops only, the arrangement being to protect a certain number of men in a few selected units, the unprotected, living under identical conditions, would thus act as controls. Special dysentery case sheets were distributed to the hospitals concerned for recording the clinical signs and symptoms of all cases of dysentery treated during the period of this investigation. The laboratory technique employed was that described by Major Manifold (1 and 2) in his Poona investigations.

(2) The form of the preparation. The Bihvaccin is prepared by the Biotherapie Company, Paris, according to the researches of Professor Besredka. It is presented in the form of dark brown tablets packed in small glass containers, three tablets per container. Three tablets represent the full prophylactic dose for an adult. Full directions are issued with each package. The following extracts are quoted —

‘Take on empty stomach one tablet of vaccine on three successive mornings. Food can be taken one hour after the ingestion of the vaccine.

After the entire dose has been taken (three vaccine tablets in three days), complete immunity is secured.

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The immunity acquired lasts for one year. In case of epidemics, it is advisable to repeat the vaccination in a few months.

No special diet is necessary.’

(3) The distribution of the vaccine. It was decided to protect one entire infantry unit and a portion of another at Poona and 300 troops at Secunderabad.

The final distribution of the vaccine was as follows —

1st Bn The Cheshire Regiment	856 doses
2nd Bn The Royal Irish Rifles	250 „
British troops, Secunderabad	294 „

(4) The method of administration of the vaccine. The selected troops were paraded by companies, on three successive mornings, soon after reveille, without having partaken of the usual ‘gun-fire’ cup of tea. The men were arranged in line, three deep. The front man of each row was handed a container of three tablets and, having helped himself to one, passed the remaining ones to the men behind him. No food was taken before one hour had elapsed.

(5) The immediate effects. The great majority experienced no after effects on swallowing the tablets. A few complained of slight abdominal discomfort. No case developed dysentery during or immediately after the administration of the vaccine.

The findings at Poona and Secunderabad will be dealt with separately

### THE POONA INVESTIGATIONS

The vaccine was distributed to the troops during the third week of June, just prior to the expected 'dysentery season' The material for this investigation was collected during the period 1st July to 31st October, 1927 During the period 43 cases of dysentery occurred amongst the British troops at Poona Of these —

36 or 83·7 per cent were bacillary in origin

4 or 9·3 per cent were protozoal in origin

3 or 7·0 per cent were unclassified

The 36 cases of definite bacillary dysentery will first be considered in this report

In 24 cases (66·7 per cent) the infecting organism was isolated, in the remaining 12 cases the typical bacillary exudate was demonstrated microscopically

The distribution of these amongst the protected and the unprotected was as follows —

	Shiga	Flexner	Bacillary exudate.	Total
Protected	2	11	9	22
Unprotected	3	8	3	14
TOTALS	5	19	12	36

### *The Incidence of Cases in the Various Units, Poona*

	Strength of units	Number of cases	Ratio per 1000
<i>1st Cheshires</i>			
Protected	856	16	18·69
Unprotected	36	1	27·78
TOTAL REGIMENT	892	17	19·06

### *2nd Ulsters*

	Strength of units	Number of cases	Ratio per 1000
Protected	250	6	24·00
Unprotected	552	7	12·68
TOTAL REGIMENT	802	13	16·21

<i>Remanning British troops</i>			
Protected	Nil		
Unprotected	1,016	6	5.90
<i>Total British troops, Poona</i>			
Protected	1,106	22	19.89
Unprotected	1,604	14	8.73
TOTAL NUMBERS B T, POONA	2,710	36	13.21

Add to these figures the unclassified cases (exudate indefinite but suggestive of bacillary dysentery)

<i>Total British troops, Poona</i>			
Protected	1,106	24	20.79
Unprotected	1,604	15	9.35
TOTAL NUMBERS B T, POONA	2,710	39	14.39

#### *Clinical Aspects of the Disease, Poona*

The first set of figures refers to cases of definite bacillary dysentery, those within brackets refer to definite plus unclassified dysenteries

Duration of symptoms in days	Protected cases		Unprotected cases	
Abdominal pain	1.29	(1.27)	1.50	(1.46)
Persistence of blood and mucus	2.14	(2.04)	1.71	(1.67)
Persistence of mucus only	3.91	(3.75)	2.71	(2.80)
Duration of fever	1.00	(0.96)	1.57	(1.46)
Highest temperature recorded	101°F		104°F	

#### THE SECUNDERABAD INVESTIGATIONS

The material for this investigation was collected during the period 1st July to 31st December, 1927. During this period 57 cases of dysentery occurred amongst the British troops stationed at Secunderabad. These cases were classified as follows —

44 or 77.0 per cent were bacillary in origin



2 or 3·5 per cent were protozoal in origin

11 or 19·5 per cent were unclassified

The report will first deal with the definite cases of bacillary dysentery

The infecting organism was isolated in 26 (59·1 per cent) of the cases, the remainder showed the typical bacillary exudate. The distribution was as follows —

	Shiga	Flexner	Bacillary exudate	Total
Protected	1	2	3	6
Unprotected	7	16	15	38
TOTALS	8	18	18	44

*The Incidence of Cases in the Various Units, Secunderabad*

	Strength of units	Number of cases	Ratio per 1,000
<i>1st Loyals</i>			
Protected	148	4	27·02
Unprotected	598	11	18·39
TOTAL REGIMENT	746	15	20·10

*1st Gordons*

Protected	117	2	17·09
Unprotected	600	14	23·33
TOTAL REGIMENT	717	16	22·31

*Remaining British troops*

Protected	29	Nil	
Unprotected	878	13	14·78
TOTAL REMAINING B T	907	13	14·33

	Strength of units	Number of cases	Ratio per 1,000
<i>Total British troops, Secunderabad</i>			
Protected	294	6	20.41
Unprotected	2,076	38	18.30
TOTAL NUMBERS B T, S'BAD	2,370	44	18.50

Add to these figures the unclassified cases—their distribution was as follows —

Among protected	1
„ unprotected	10

*Total British troops, Secunderabad*

Protected	294	7	23.80
Unprotected	2,076	48	23.12
TOTAL NUMBERS B T, S'BAD	2,370	55	23.21

*Clinical Aspects of the Disease, Secunderabad*

Duration of symptoms in days	Protected cases	Unprotected cases
Abdominal pain	1.50 (1.83)	1.76 (1.73)
Persistence of blood and mucus	2.50 (2.43)	2.95 (2.59)
Persistence of mucus only	3.83 (4.16)	3.69 (3.67)
Duration of fever	1.00 (1.43)	1.23 (1.19)
Highest temperature recorded	103°F	102.6°F

POONA AND SECUNDERABAD FINDINGS COMBINED

*A* Definite bacillary dysentery cases only

	Strength	Number of cases	Ratio per 1,000
Protected	1,400	28	20.00
Unprotected	3,680	52	14.10
TOTALS	5,080	80	15.75

## B. Definite bacillary dysentery cases plus unclassified cases

	Strength	Number of cases	Ratio per 1,000
Protected	1,400	31	22.14
Unprotected	3,680	63	17.12
TOTALS	5,080	94	18.50

## Clinical Findings, Poona and Secunderabad, Combined

Duration of symptoms in days	Protected cases		Unprotected cases	
Abdominal pain	1.39	(1.55)	1.63	(1.59)
Persistence of blood and mucus	2.32	(2.23)	2.35	(2.13)
Persistence of mucus only	3.87	(3.95)	3.20	(3.23)
Duration of fever	1.00	(1.19)	1.44	(1.32)
Highest temperature recorded	103°F		104°F	

## SUMMARY

(1) The efficacy of anti-dysenteric Bilivaccin as a prophylactic against bacillary dysentery was tried out among the British troops stationed at Poona and Secunderabad. At Poona with a British garrison of 2,710 men, 1,106 (41.0 per cent) were protected, while at Secunderabad with a British garrison of 2,370 men, 294 (12.7 per cent) were protected. The total number protected was 1,400 out of a total of 5,080 (27.6 per cent).

(2) Alternative records are presented, the first dealing with definite cases of bacillary dysentery, the second including unclassified cases in addition to definite cases.

(3) A few records, showing the clinical aspect of the disease among the protected and the unprotected, are also presented.

## CONCLUSIONS

Oral Bilivaccin failed as a prophylactic against bacillary dysentery during our investigations at Poona and Secunderabad.

The clinical course of the disease did not appear to be modified in individuals protected by the vaccine.

We have to thank Major Pottinger, M.C. R.A.M.C., and Major Stevenson, R.A.M.C., for supplying us with clinical records of all cases of dysentery treated.

J. MR

by them during the period of this investigation, at Poona and Secunderabad respectively

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# A SHORT NOTE ON THE AGGLUTINABILITY OF T A B EMULSIONS PREPARED BY VARIOUS METHODS

BY

LIEUT S M K MALLICK, M R C S , L R C P , D P H , I M S

AND

ASSISTANT SURGEON M G COOMBES, D T M , I M D ,

*Central Research Institute, Kasauli*

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THE validity of the old routine agglutination test of enteric fevers with dead carbolized and formalized emulsions has recently been questioned by many workers amongst whom the work of Felix and Oltzki(1) deserves special consideration. The discovery of rough and smooth variants(2) of various organisms produced in nature by unfavourable circumstances and artificially by various excitants has led to the study of their differential characters including agglutinative reactions.

Two varieties of agglutinations have been recognized for most of the organisms, the stabilotropic and labilotropic, each representing the O or the heat stabile (100°C for half an hour), and H or the heat labile antigen. Differences not only in the type of agglutination (flocculent, granular and mixed) but in the effect of heat and various preservatives on the emulsions of the organism have been elucidated, and consequently a double experiment for each Widal test in routine work was advised with emulsions preserved with 1 per cent alcohol for O and 0.5 per cent carbolic acid for H agglutinin.

As a good number of Widal tests are done here, we thought it worth while to study the effect of various methods of preparation of the emulsion on the agglutinability of *B. typhosus* and *B. paratyphosus* A and B. Agglutination being dependent on various factors such as the reaction of the medium (pH), its sugar content, humidity, the age of the growth and the technique employed, it was decided to keep all these factors constant and to vary only the treatment of the emulsion and to see how this affected the results.

High titre serum was obtained from a rabbit given 1 cc, 2 cc, 4 cc and 8 cc of the stock T A B vaccine at 3 days' interval and bled a week

	Strains	Non-heated						Heated 56°C ½ hr						Heated 96.5°C ½ hr									
		1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Control	Type of agglutination	Mixed	"	"	"	"	"	Granular	"	"	"	"	"
Non-heated	<i>B typhosus</i> (Enteric Depôt)	++	++	++	++	++	++	++	++	++	Flocculent	+	+	+	+	+	+	+	+	+	+	+	+
	<i>B typhosus</i> (Rawlings)	++	++	++	++	++	++	++	++	+	"	+	+	+	+	+	+	+	+	+	+	+	+
	<i>Para A</i> (Enteric Depôt)	++	++	++	+	+	+	+	+	+	"	+	+	+	+	+	+	+	+	+	+	+	+
	<i>Para A</i> (Mears)	++	++	++	++	++	++	++	++	+	"	+	+	+	+	+	+	+	+	+	+	+	+
	<i>Para B</i> (Enteric Depôt)	++	++	++	++	++	++	++	++	+	"	+	+	+	+	+	+	+	+	+	+	+	+
	<i>Para B</i> (Rowland)	++	++	++	++	++	++	++	++	+	"	+	+	+	+	+	+	+	+	+	+	+	+
Heated 56°C ½ hr	<i>B typhosus</i> (Enteric Depôt)	++	++	++	++	++	++	++	++	++	Mixed	+	+	+	+	+	+	+	+	+	+	+	+
	<i>B typhosus</i> (Rawlings)	++	++	++	++	++	++	++	++	++	"	+	+	+	+	+	+	+	+	+	+	+	+
	<i>Para A</i> (Enteric Depôt)	++	++	++	++	+	+	+	+	+	"	+	+	+	+	+	+	+	+	+	+	+	+
	<i>Para A</i> (Mears)	++	++	++	++	++	++	++	++	+	"	+	+	+	+	+	+	+	+	+	+	+	+
	<i>Para B</i> (Enteric Depôt)	++	++	++	++	++	++	++	++	+	"	+	+	+	+	+	+	+	+	+	+	+	+
	<i>Para B</i> (Rowland)	++	++	++	++	++	++	++	++	+	"	+	+	+	+	+	+	+	+	+	+	+	+
Heated 96.5°C ½ hr	<i>B typhosus</i> (Enteric Depôt)	++	++	++	++	++	++	++	++	++	Granular	+	+	+	+	+	+	+	+	+	+	+	+
	<i>B typhosus</i> (Rawlings)	++	++	++	++	++	++	++	++	++	"	+	+	+	+	+	+	+	+	+	+	+	+
	<i>Para A</i> (Enteric Depôt)	++	++	++	++	++	++	++	++	++	"	+	+	+	+	+	+	+	+	+	+	+	+
	<i>Para A</i> (Mears)	++	++	++	++	++	++	++	++	++	"	+	+	+	+	+	+	+	+	+	+	+	+
	<i>Para B</i> (Enteric Depot)	++	++	++	++	++	++	++	++	++	"	+	+	+	+	+	+	+	+	+	+	+	+
	<i>Para B</i> (Rowland)	++	++	++	++	++	++	++	++	++	"	+	+	+	+	+	+	+	+	+	+	+	+

Type of agglutination	Control						Control					
	Flocculent						Flocculent					
Carbolized 5 per cent	<i>B typhosus</i> (Enteric Depot)	++	++	++	++	++	++	++	++	++	++	++
	<i>B typhosus</i> (Rawlings)	++	++	++	++	++	++	++	++	++	++	++
	<i>Para A</i> (Enteric Depot)	++	++	++	++	++	++	++	++	++	++	++
	<i>Para I</i> (Mears)	++	++	++	++	++	++	++	++	++	++	++
	<i>Para B</i> (Enteric Depot)	++	++	++	++	++	++	++	++	++	++	++
	<i>Para B</i> (Rowland)	++	++	++	++	++	++	++	++	++	++	++
Formalized 2 per cent	<i>B typhosus</i> (Enteric Depot)	++	++	++	++	++	++	++	++	++	++	++
	<i>B typhosus</i> (Rawlings)	++	++	++	++	++	++	++	++	++	++	++
	<i>Para I</i> (Enteric Depot)	++	++	++	++	++	++	++	++	++	++	++
	<i>Para I</i> (Mears)	++	++	++	++	++	++	++	++	++	++	++
	<i>Para B</i> (Enteric Depot)	++	++	++	++	++	++	++	++	++	++	++
	<i>Para B</i> (Rowland)	++	++	++	++	++	++	++	++	++	++	++

++++, ++, +, — = varying degrees of agglutination, estimated with the naked eye ± = traces, estimated by means of a magnifying lens

after the last injection. It is rather disappointing that we could not kill the organisms with 1 to 5 per cent alcohol, so the following emulsions were used —

- 1 Living emulsion in normal saline
- 2 Emulsion heated to 96.5°C for half an hour
- 3 Emulsion heated to 56°C for half an hour
- 4 Carbolyzed emulsion (0.5 per cent)
- 5 Formalized emulsion (0.2 per cent)

Colonel Harvey's technique of agglutination was employed (3). The agglutination titres reacted with the different emulsions were as follows, the titre given being the limit of naked eye agglutination observed in each case

Method of preparation	<i>B. typhosus</i> , Enteric Depôt	<i>B. typhosus</i> , Rawlings	<i>Para A</i> , Enteric Depôt	<i>Para A</i> , Mears	<i>Para B</i> , Enteric Depôt	<i>Para B</i> , Rowland
Non-heated	1/2560	1/5120	1/320	1/640	1/5120	1/5120
Heated 56°C	1/640	1/1280	1/160	1/320	1/1280	1/5120
Heated 96.5°C	1/320	1/640	1/40	1/80	1/640	1/2560
Carbolyzed	1/1280	1/5120	1/160	1/320	1/1280	1/2560
Formalized	1/2560	1/5120	1/160	1/320	1/2560	1/5120

The attached table shows the actual agglutination readings

#### SUMMARY

- 1 Non-heated emulsions of organisms in normal saline give the best results
- 2 Heating up to 56°C and 96.5°C for half an hour gives rise to a marked decrease in the agglutinability of the organisms
- 3 Emulsions preserved with formalin give better results than the carbolyzed ones

4 The agglutinin response of the only rabbit used in our experiments was in the following order *B. paratyphosus B*, *B. typhosus* and *B. paratyphosus A*, but it does not follow that this would hold good in all cases

Our thanks are due to Major L. A. P. Anderson, R.M.S., for his guidance and supervision throughout the above experiments

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A SHORT NOTE ON THE AGGLUTINOGENETIC POWERS OF  
THE ROUGH AND THE SMOOTH VARIANTS OF  
*B TYPHOSUS* AND THEIR MUTUAL  
IMMUNOLOGICAL RELATIONSHIP

Part I.

BY

LIEUT S M K MALLICK, M R C S, L R C P, D P H, I M S,

AND

ASSISTANT SURGEON M G COOMBES, D T M, I M D,

*Central Research Institute, Kasauli*

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MICROBIC dissociation is by now a universally known phenomenon and has been proved to occur in many bacterial species, both accidentally and as a result of various excitants such as are employed in the laboratory for experimental purposes

A great deal of work has been done in recent years to investigate the antigenic composition of the smooth and rough variants of the enteric group of organisms by Arkwright, Felix and Olitzki, Gardner, White, and a number of other eminent research workers and many different opinions have been expressed by them on this subject. The experiments described in this note were started to see, how by ordinary laboratory methods one can differentiate the various agglutinins produced by the rough and smooth variants of *B typhosus* and also to ascertain their protective values when used for prophylactic vaccines

The two variants of *B typhosus*, rough (T R) and smooth (T S) respectively, were kindly given to us by Major L. A. P. Anderson, I M S, who had imported them from England. We have studied their morphological, cultural, biochemical and serological characters. The rough variant was tested also for its 'roughness' by its growth on Douglas agar, broth and auto-agglutination in normal saline solution. Both the variants were motile.

Two formalized emulsions were made of each of the variants (one of each of these was heated at 100°C for half an hour before being formalized) and

TABLE  
Dilutions of sera

Sera	Emulsions	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Control	Type of agglutination
T R Heated	T R Non heated	++	++	++	++	++	++	++	++	++	++	++	Mixed Granular
	T R Heated	++	++	++	++	++	++	++	++	++	++	++	Floccular Granular
	T S Non heated	++	++	++	++	++	++	++	++	++	++	++	Floccular Granular
	T S Heated	++	++	++	++	++	++	++	++	++	++	++	Floccular Granular
T S Non-heated	T R Non-heated	++	++	++	++	++	++	++	++	++	++	++	Floccular Granular
	T R Heated	++	++	++	++	++	++	++	++	++	++	++	Floccular Granular
	T S Non heated	++	++	++	++	++	++	++	++	++	++	++	Floccular Granular
	T S Heated	++	++	++	++	++	++	++	++	++	++	++	Floccular Granular
T R Non-heated	T R Non-heated	++	++	++	++	++	++	++	++	++	++	++	Floccular Granular
	T R Heated	++	++	++	++	++	++	++	++	++	++	++	Floccular Granular
	T R Alcoholized	++	++	++	++	++	++	++	++	++	++	++	Mixed Granular
	T S Alcoholized	++	++	++	++	++	++	++	++	++	++	++	Mixed Granular
T S Heated	T R Non-heated	++	++	++	++	++	++	++	++	++	++	++	Mixed Granular
	T R Heated	++	++	++	++	++	++	++	++	++	++	++	Floccular Granular
	T S Non-heated	++	++	++	++	++	++	++	++	++	++	++	Floccular Granular
	T S Heated	++	++	++	++	++	++	++	++	++	++	++	Floccular Granular

++++, ++, +, ±, + = varying degrees of agglutination, estimated with the naked eye (±) = traces, estimated by means of a magnifying lens

In determining the type, we have taken into consideration the predominant character of agglutinations. All emulsions were standardized to contain 1 mg of dried bacterial substance per c.c.

high titre sera were prepared against each by intravenous injection of 0.1 mg, 0.2 mg, 0.4 mg and 0.8 mg in rabbits, at weekly intervals, the animals being bled 10 days after their last injection

A number of experiments were put up with each serum, the Widal sets incubated in a water bath at 55°C for 4 hours and the results read overnight, Harvey's technique of agglutination being employed

The results of these experiments are given in the preceding Table

### CONCLUSIONS

1 Each variant gives rise to two types of agglutinins, one corresponding to its heat stable and the other to its heat labile antigen

2 Although theoretically, the heating of the emulsions of either of these variants for 30 minutes at 100°C should destroy the heat labile antigen and when these emulsions are injected into rabbits should produce agglutinins corresponding to their heat stable antigen only, yet we do get with the injection of heated emulsions a certain amount of agglutinins corresponding to the heat labile antigens

3 Agglutinins produced by heated emulsions of the two variants react to a lesser extent with the heat labile antigen of the same variant, but to a greater extent with the heat labile antigen of the other variant

4 Heated emulsions show the heat stable antigen of each variant to a lesser extent than alcoholized emulsions, which also show a better and more distinct difference in the rough and smooth serological reactions

5 The rough variant also contains a specific heat stable antigen like the smooth variant as differentiated by alcoholized emulsions

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# THE RELATIVE TOXICITY AND IMMUNIZING VALUE OF HAFFKINE'S PLAGUE PROPHYLACTIC AND OTHER ANTI-PLAGUE VACCINES COMPARED \*

BY

B P B NAIDU, M D (Edin ), M H , D P H , D T M (L'pool),

AND

JAMEDAR SHAMSHER JUNG, I M D,†

*Haffkine Institute, Bombay*

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PROPHYLACTIC vaccines against plague which are now in use differ in the methods of their manufacture and represent three distinct types of anti-plague vaccines

To the first type belongs Haffkine's plague prophylactic, manufactured at the Haffkine Institute, Bombay. This vaccine is a broth culture of highly virulent plague bacilli grown for a period of six to eight weeks in the dark and sterilized by heat. It is a whole vaccine composed of the culture medium together with the bacillary bodies and the products of their metabolism. The immunizing dose of this vaccine for man is 4 c c.

Haffkine's plague prophylactic has been extensively employed in India and the statistics regarding its protective value in man have been carefully collected from time to time which go to show that the incidence of the disease among the inoculated is considerably reduced and the case mortality among the inoculated who subsequently contract plague is reduced by at least one-half (Table I).

In order to estimate the potency of anti-plague vaccines in laboratory animals, it is necessary to fix upon a suitable test dose for infection. The test virus

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\* Paper read before the Indian Science Congress, 1929

† This able research worker has since died from pneumonic plague contracted during the execution of his duty. F P Mackie, Lieutenant-Colonel, I M S, Director, Haffkine Institute, February, 1929

should exhibit a uniform degree of virulence throughout a series of experiments that may extend over many months. Agar or broth cultures are open to objection. Their virulence depends on the source from which the seed culture has been obtained, the constitution of the medium, and the duration of incubation.

TABLE I

	Population	Number of attacks	Ratio per 100	Number of deaths	Ratio per 100	Percentage survivors from attacks
Figures compiled from the report of Bannerman (1897-1900) and from the Annual Reports of the Bombay Bacteriological Laboratory (1902-1916)	Un-inoculated	59,668	1.8	50,735	1.5	14.9
	Inoculated	4,126	0.21	1,620	0.08	60.7
Figures taken from statistics of inoculation in the town of Ilkal, in Bijapur District, where a careful house to house inquiry was made. Report of the Bombay Bacteriological Laboratory for the years (1915-1916)	Un-inoculated	20	51.2	19	48.71	5.0
	Inoculated	4	6.34	1	1.58	75.0
Figures taken from the Annual Report of the Director of Public Health, Madras, 1926	Un-inoculated	26	1.2	21	0.99	19.2
	Inoculated	62	0.32	13	0.07	79.0

At the Haffkine Institute, for many years, the virus used has been the spleen of a rat dying of plague. Strains are obtained from human cases and are passed from rat to rat so long as they continue to kill the rat within four days. Our practice in these experiments has been to use a uniform dose of 0.003 milligram of such a spleen freshly removed soon after death. A portion of the spleen is weighed and ground in a mortar with broth, this is diluted with broth till 1 c.c. of it contained 0.003 milligram of the spleen pulp. Administered subcutaneously to rats in our various control experiments, about 94 per cent of rats died of plague within 15 days of infection, further, the daily mortality rate in rats following on the injection of this dose, closely corresponded with the daily mortality rate in rats infected by the cutaneous method (Table II).

With this test dose, we have carried out experiments to determine the susceptibility of other laboratory animals, namely, guinea-pigs and rabbits. The results go to show that they are highly susceptible to infection, and that in these also, the disease is characterized by manifestations of a general infection and hæmorrhagic inflammatory processes of the internal organs (Table III).

TABLE III

*Mortality following on the subcutaneous infection with 0.003 milligram of spleen of a rat died of acute plague in guinea-pigs and rabbits compared with that of rats*

	Number of experiments	Number of animals used	Deaths in 7 days	Percentage deaths in 7 days	Deaths in 10 days	Percentage deaths in 10 days	Deaths in 15 days	Percentage deaths in 15 days
Guinea-pigs	12	40	30	75.0	35	87.5	38	95.0
Rabbits	11	47	35	74.4	36	76.6	39	83.0
Rats		1,629	1,476	90.6	1,516	93.0	1,535	94.2

Furthermore, experiments carried out in rats, guinea-pigs and rabbits with increasing dilutions of the test dose, namely, 0.003 milligram of plague spleen, seem to indicate that the mortality in these animals following on experimental infection, is dependent not on the actual number of organisms employed but on their virulence (Table IV).

In order to estimate the toxicity and immunizing value of Haffkine's plague prophylactic, it is necessary to fix upon a suitable dose for the experimental animals. It is generally held that the immunizing dose for rats is either 1/16th or 1/8th the human dose. The results of our experiments with the prophylactic

TABLE II  
Daily mortality among *Madras rattus rattus* following on experimental infection with plague

Mode of infection	During the years	Number of rats used	Percentage of deaths in 7 days							Percentage deaths in 10 days			Percentage deaths in 15 days					Total plague deaths	Percentage deaths in 15 days
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
By cutaneous passage	1923—1925	283	0	29	113	95	22	3	2	(93.3)	2	1	1	0	0	0	0	268	94.7
	1926	539	0	38	271	145	37	12	7	(94.6)	1	0	2	0	1	1	0	515	95.5
	1927	539	0	26	227	182	53	13	2	(93.3)	3	0	0	0	0	0	0	506	93.8
By subcutaneous injection of 0.003 miligram of plague-spleen	1923—1925	1,361	0	93	611	422	112	28	11	(93.8)	6	1	3	0	1	1	0	1,289	94.7
		541	0	11	225	145	50	25	18	(87.6)	2	7	4	0	0	2	5	494	91.3
	1926	575	0	35	210	165	69	31	18	(91.8)	6	5	5	2	1	1	2	550	95.6
	1927	513	0	8	165	173	78	37	13	(92.4)	5	3	3	3	2	0	1	491	95.7
		1,629	0	54	600	483	197	93	49	(90.6)	13	15	12	5	3	3	8	1,535	94.2



TABLE IV  
The relative susceptibility of Madras rats, guinea-pigs and rabbits to experimental infection with the spleen of a rat died of acute plague in decreasing doses

Test dose employed	MADRAS RATS				GUINEA-PIGS				RABBITS			
	Number of experi- ments	Number of rats infected	Deaths in 15 days	Percentage sur- vivors	Number of experi- ments	Number of guinea- pigs infected.	Deaths in 15 days	Percentage survi- vors	Number of experi- ments	Number of rabbits infected	Deaths in 15 days	Percentage survi- vors
Test dose of plague (0.003 mg.)	56	600	574	4.3	12	40	38	50	11	47	39	17.0
1/10th	14	140	125	10.7								
1/100th	14	140	123	12.1					3	15	14	66
1/1,000th	10	100	94	6.0								
1/10,000th	10	100	94	6.0					3	15	13	13.3
1/100,000th	10	100	94	6.0								
1/1,000,000th	10	100	88	12.0	2	20	20	0.0	9	26	22	15.4
1/10,000,000th	10	100	97	3.0								
1/100,000,000th	10	100	95	5.0	2	20	20	0.0	1	2	2	0.0
1/1,000,000,000th	3	40	38	5.0								
1/10,000,000,000th	3	40	40	0.0	2	20	19	5.0	1	2	2	0.0
1/100,000,000,000th	3	40	38	5.0	2	20	20	0.0	1	2	2	0.0
1/1,000,000,000,000th	3	40	36	10.0	2	20	19	5.0	1	2	2	0.0

in these doses, go to show that there is no appreciable difference either on the toxicity or in the immunizing value in rats in these doses (Table V)

TABLE V

*The toxicity and immunizing value of 0.25 cc (1/16th the human dose) and 0.5 cc (1/8th the human dose) of Haffkine's Plague Prophylactic compared*

Brew Nos	IMMUNIZING DOSE = 0.25 cc						IMMUNIZING DOSE = 0.5 cc					
	Rats immunized	Deaths in 7 days	Percentage toxic deaths	Survivors infected	Deaths in 15 days	Percentage immunity	Rats immunized	Deaths in 7 days	Percentage toxic deaths	Survivors infected	Deaths in 15 days	Percentage immunity
148	30	1	3.3	29	14	51.7	30	1	3.3	29	8	72.4
152	30	2	6.6	28	22	21.4	30	5	16.6	25	17	32.0
155	30	1	3.3	29	22	24.1	30	1	3.3	29	18	31.0
156	30	0	0.0	30	15	50.0	30	2	6.6	28	13	53.5
159	30	3	10.0	27	23	14.8	30	3	10.0	27	22	18.5
184	30	1	3.3	29	23	20.7	30	1	3.3	29	20	31.0
186	30	0	0.0	30	24	20.0	30	0	0.0	30	19	36.6
190	30	0	0.0	30	23	23.3	30	0	0.0	30	22	26.6
194	30	0	0.0	30	21	30.0	30	1	3.3	29	22	24.1
196	30	1	3.3	29	22	24.1	30	0	0.0	30	18	40.0
10 Brews	300	9	3.0	291	209	28.1	300	14	4.6	286	179	37.0

Similar experiments with the prophylactic in guinea-pigs seem to indicate that in these two doses, the vaccine has no toxic effect in guinea-pigs and that the immunizing value of the vaccine is considerably below that which is obtained in rats (Table VI)

As plague is primarily a disease of rats, rats are, therefore, the animals of choice for experimental study with plague. But rats in an area like the city of Bombay where plague has existed for several years, become relatively immune to plague, whereas rats from an area like the city of Madras, where plague has not so far obtained a footing, are more susceptible and have a very uniform rate of mortality to experimental infection (Table VII)

TABLE VI

*The toxicity and immunizing value of 0.25 cc (1/16th the human dose) and 0.5 cc. (1/8th the human dose) of Haffkine's Plague Prophylactic compared in guinea-pigs*

Brew Nos	IMMUNIZING DOSE = 0.25 cc						IMMUNIZING DOSE = 0.5 cc						CONTROLS	
	Guinea-pigs immunized	Deaths in 7 days	Percentage toxic deaths	Survivors infected	Deaths in 15 days	Percentage immunity	Guinea-pigs immunized	Deaths in 7 days	Percentage toxic deaths	Survivors infected	Deaths in 15 days	Percentage immunity	Guinea-pigs infected	Deaths in 15 days
101	10	0	0.0	10	8	20.0	10	0	0.0	10	6	40.0	2	1
110	10	0	0.0	10	8	20.0	10	0	0.0	10	7	30.0	2	2
112	10	0	0.0	10	8	20.0	10	0	0.0	10	8	20.0	2	1
126	10	0	0.0	10	10	0.0	10	0	0.0	10	6	40.0	2	2
136	10	0	0.0	10	10	0.0	10	0	0.0	10	7	30.0	2	2
144	10	0	0.0	10	10	0.0	10	0	0.0	10	10	0.0	2	2
146	10	0	0.0	10	10	0.0	10	0	0.0	10	10	0.0	2	2
148	10	0	0.0	10	10	0.0	10	0	0.0	10	10	0.0	2	2
152	10	0	0.0	10	10	0.0	10	0	0.0	10	10	0.0	2	2
159	10	0	0.0	10	10	0.0	10	0	0.0	10	10	0.0	2	2
10 Brews	100	0	0.0	100	94	6.0	100	0	0.0	100	84	16.0	20	18 (10 per cent)

TABLE VI(a)  
Relative immunizing value of Haffkine's Plague Prophylactic in guinea-pigs and in rats compared

Brew Nos	IMMUNIZING DOSE = 0.5 cc				IMMUNIZING DOSE = 0.5 cc				CONTROLS				CONTROLS			
	Guinea-pigs immunized	Survivors infected	Deaths in 15 days	Percentage immunity	Rats immunized	Survivors infected	Deaths in 15 days	Percentage immunity	Guinea-pigs infected	Deaths in 15 days	Percentage immunity	Rats infected	Deaths in 15 days	Percentage survivors		
101	10	10	6	40.0	60	55	28	49.0	2	1	50.0	10	10	0.0		
110	10	10	7	30.0	60	58	35	39.6	2	2	0.0	10	10	0.0		
112	10	10	8	20.0	60	55	40	27.2	2	1	50.0	10	10	0.0		
126	10	10	6	40.0	60	57	32	43.8	2	2	0.0	10	9	10.0		
136	10	10	7	30.0	60	52	31	40.3	2	2	0.0	10	10	0.0		
144	10	10	10	0.0	60	58	43	25.8	2	2	0.0	10	9	10.0		
146	10	10	10	0.0	60	55	22	60.0	2	2	0.0	10	10	0.0		
148	10	10	10	0.0	30	26	19	26.9	2	2	0.0	10	9	10.0		
152	10	10	10	0.0	30	25	17	32.0	2	2	0.0	10	10	0.0		
159	10	10	10	0.0	60	49	24	51.0	2	2	0.0	10	10	0.0		
10 Brews	100	100	84	16.0	540	490	291	40.6	20	18	10.0	100	97	3.7		

TABLE VII  
The relative susceptibility of *Madras rats* (*rattus rattus*) and *Bombay rats* (*rattus rattus*) to experimental infection with plague

Month	1926				1927				1928			
	MADRAS RATS		BOMBAY RATS		MADRAS RATS		BOMBAY RATS		MADRAS RATS		BOMBAY RATS	
	Rats infected	Percentage plague deaths	Rats infected	Percentage plague deaths	Rats infected	Percentage plague deaths	Rats infected	Percentage plague deaths	Rats infected	Percentage plague deaths	Rats infected	Percentage plague deaths
January					50	90.0	116	18.2	98	94.8	120	23.3
February					50	94.0	119	19.4	84	92.8	120	24.1
March					60	95.0	120	27.5	120	97.5	120	33.3
April					50	92.0	99	19.2	95	91.5	120	15.8
May	20	90.0	50	26.0	50	100.0	120	21.7	76	97.3	100	14.0
June	30	93.4	50	16.0	40	97.5	119	16.9				
July	40	90.0	49	6.2	30	96.7	120	14.2				
August	30	96.7	200	6.0	40	97.5	120	10.0				
September	30	93.4	149	20.9	50	100.0	120	10.9				
October	110	99.1	113	35.1	30	93.4	118	27.2				
November	100	94.0	117	18.9	20	100.0	119	22.7				
December	110	95.5	120	21.0	50	96.0	117	10.3				
Total	470	95.1	848	17.6	520	95.7	1,407	18.1	473	94.8	580	22.4



Therefore, in these experiments we have employed rats imported from Madras and have used 1/8th the human dose of the anti-plague vaccine for immunization

It has been shown(1) that following on the inoculation of Haffkine's prophylactic in rats, immunity begins to develop, and reaches its maximum in three to five days. We have carried out experiments to estimate the amount of immunity conferred by the prophylactic, by allowing an interval of seven days and of fourteen days between prophylactic inoculation and infection. The results do not seem to indicate that a greater degree of immunity follows by increasing the interval to fourteen days (Table VIII)

To estimate the degree of protection conferred by anti-plague vaccine in rats, we have allowed an interval of fifteen days after infection and calculated the percentage protection or immunity on the number of survivors at the end of that period

During the years 1923, 1924 and 1925 we have carried out several experiments on different brews of the prophylactic to estimate their toxicity and protective power in rats. The results of these experiments showed that it is possible to standardize the potency of the different brews of the prophylactic by this method, and since 1926, every brew of the prophylactic has been tested for its potency on rats and only such brews which yielded a sufficient degree of protection have been issued for general use (Table IX)

TABLE IX

*The average potency of the different brews of Haffkine's plague prophylactic manufactured at Parel, Bombay*

Years	Number of Brews examined	Number of experiments	Number of rats inoculated	Survivors percentage immunity	CONTROL	
					Rats infected	Survivors percentage immunity
1923—1925	29	49	1,015	33.9	482	6.7
1926	62	82	3,350	29.8	360	6.6
1927	48	72	3,470	36.4	338	5.4
To the end of September 1928	30	45	1,440	36.6	370	7.0
TOTAL	169	248	9,275	34.2	1,550	6.4

Therefore, the protective value of Haffkine's plague prophylactic both for man and rat is established

The second type of anti-plague vaccine is the agar-grown vaccines manufactured in Germany (I G Farbenindustrie Aktiengesellschaft, Hoechst Am

Mam), in France (Pasteur Institute, Paris), and in England (Lister Institute, London) These are prepared from plague cultures grown on a solid medium for two or three days, washed with normal saline, standardized by either the opacity or the counting method, and sterilized by heat or by an antiseptic These are derived vaccines, and contain only the bacillary bodies Their immunizing dose for man is 1 c c

Agar-grown vaccines have been employed in French Possessions, in Dutch East Indies, in United States, in South Africa, and in the Gold Coast Statistical evidence of their protective value in man are meagre It is generally held that agar-grown vaccines are less toxic than Haffkine's prophylactic, their protective dose for man is much smaller than that of the prophylactic In the printed directions accompanying the anti-plague vaccines issued by the German, French, and English manufacturers, attention is drawn to the fact that the injection of these vaccines in man is usually followed by a local reaction appearing at the site of injection and that the local reactions are frequently associated with general symptoms, such as, malaise, fatigue, and fever lasting for one or two days

In view of the fact that the preparation of agar-grown vaccines involves considerably less time compared with that of the manufacture of the prophylactic which extends over many weeks, we have carried out comparative experiments to estimate the relative toxicity and protective value of anti-plague vaccines now in common use The results of these experiments, so far, go to show that agar-grown vaccines, even in the doses recommended for man, produce a lower degree of protection in rats as compared with Haffkine's prophylactic in a dose of 0.5 c c which is one-eighth the human dose (Table X)

To the third type of vaccine belongs Lustig's plague nucleo-proteid, it is prepared from agar cultures, the growth is washed in saline, the bacillary bodies are dissolved by an alkali, and the nucleo-proteid is precipitated with a weak acid, the precipitate is collected, dried, and stored in the dark This vaccine is sent out in bottles containing 0.04 gramme of the nucleo-proteid dissolved in 21 c c of an alkaline solution, and 7 c c is the prophylactic dose recommended for man It is a derived vaccine containing only the nucleo-proteid which is the immunizing antigen

Here also, statistical evidence as to its protective value in man is not available We have obtained a supply of this vaccine through Prof. Lustig and have carried out experiments in rats using one-eighth the human dose for immunization The results seem to indicate that in this dose this vaccine produces in rats a degree of immunity comparable with that obtained by the use of Haffkine's plague prophylactic in one-eighth the human dose, namely, 0.5 c c (Table XI)

As one of our colleagues at the Haffkine Institute, Dr. Avari, has succeeded in isolating a strain of bacteriophage from rats which brings about the lysis of a culture of plague in less than two hours, we have carried out experiments with this 'anti-pestiphage' to estimate the degree of protection it conferred in rats in a dose of 0.5 c c The results seem to indicate that this bacteriophage has some protective value (Table XII)



TABLE X  
*Relative toxicity and immunizing value of agar-grown vaccines and Haffkine's Prophylactic compared*

Dates of experiments	Vaccines examined	Immunizing dose c.c.	Number of experiments	Rats unimmunized	Deaths in 7 days	Percentage toxic deaths	Survivors infected	Deaths in 15 days	Percentage immunity
14.5.28	Lister	0.2	2	90	0	0.0	90	82	88
1.6.28	Pasteur	0.2	2	90	1	1.1	89	77	134
	Haffkine (181, 208)	0.5	2	90	1	1.1	89	59	337
	Controls						20	18	100
18.6.28	Lister	0.4	3	90	4	4.4	86	81	59
2.7.28	Pasteur	0.4	3	90	1	1.1	89	74	168
20.8.28	Haffkine (211, 222 and 257)	0.5	3	90	1	1.1	89	64	280
	Controls						30	27	100
27.8.28	Lister	1.0	4	120	6	5.0	114	107	61
	Pasteur	1.0	4	120	4	3.3	116	102	120
	German	1.0	4	150	4	2.6	146	143	20
	Haffkine (260, 267, 268, and 273)	0.5	4	120	6	5.0	114	74	351
	Controls			.			40	39	25

TABLE XI  
Relative toxicity and immunizing value of Lustig's Nucleo-proteid and Haffkine's Prophylactic compared

Dates of experiments	Vaccines examined	Immunizing dose c c	Number of experiments	Rats immunized	Deaths in 7 days	Percentage toxic deaths	Survivors infected	Deaths in 15 days	Percentage immunity
14-5-28	Lustig	0.9	4	150	6	4.0	144	90	37.5
1-6-28 18-6-28 9-7-28	Haffkine (181, 208, 211, 222)	0.5	4	150	2	1.3	148	97	33.1
	Controls	.			.		40	38	50

TABLE XII  
Relative toxicity and immunizing value of Avari's 'Anti-Pestiplage' and Haffkine's Prophylactic compared

Dates of experiments	Vaccines examined	Immunizing dose c c	Number of experiments	Rats immunized	Deaths in 7 days	Percentage toxic deaths	Survivors infected	Deaths in 15 days	Percentage immunity
1-6-28	Avari	0.5	3	90	3	3.3	87	74	150
18-6-28 9-7-28	Haffkine (208, 211 and 222)	0.5	3	90	1	1.1	89	57	360
	Controls	..					30	29	33

### SUMMARY

1 The method which is now in use at the Haffkine Institute for standardizing the prophylactic is considered suitable for estimating the potency of anti-plague vaccines

2 Haffkine's prophylactic in a dose of 0.5 c.c. confers in rats a percentage immunity of about 35 against an infection with a large dose of highly virulent plague bacilli

3 Agar-grown vaccines of 'Farbenindustrie, Hoechst,' of the Pasteur Institute, Paris, and of the Lister Institute, London, confer a lower degree of protection in rats even when employed in doses recommended for man as compared with 0.5 c.c. of Haffkine's prophylactic

4 Lustig's nucleo-proteid produces a degree of immunity in rats comparable with that of Haffkine's prophylactic

5 Avari's 'anti-pestiphage' confers some degree of protection in rats in the dose employed against subsequent infection with plague

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# *A AITKENII* JAMES IN VIZAGAPATAM AGENCY, MADRAS PRESIDENCY

BY

A K ADHIKARI, M B, F R S T M & H (Lond),  
*Assistant Surgeon on Malaria Investigation, B N Railway*

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IN spite of various records of Anopheline mosquitoes in the Madras Presidency none of these, as will be seen by a reference to the distribution given by Covell (1927), indicate the existence of *A aitkeni* in the Vizagapatam Agency, or indeed anywhere in the Indian Peninsula in a wide tract lying between Chota Nagpur in the north-east and the Nilgiris in the south

The survey and construction of some portions of the Raipur-Vizianagram Railway have been found extremely difficult on several previous occasions owing to local hyper-endemic malarial conditions and Mr Senior White, the Malaria-logist, and his Assistant, the writer, have been engaged on malaria investigation and protection for engineering staff and coolie camps along this route, especially in district No 2 of the construction which covers the greatest extent of its length During the absence of the malaria-logist in 1927, and whilst in charge of the malaria control work and in the course of his usual routine surveys the writer came across *A aitkeni* in one of the streams in this district

It was detected first in 1928 in the first part of January, and was again found in early February On both occasions quite a number of *A aitkeni* larvæ were collected from the same 'gedda' (stream) The identification of the second finding was supported by Dr Speedy After Mr Senior White rejoined the Railway in 1928, the full description of the larvæ and the breeding-place were reported to him during his inspection of the camps, on which he confirmed the correctness of our finds

(1) Geographical situation in the Vizagapatam Agency 54 miles north from the Rail-head, Parvatipuram Station, near a small village called Illsamuthi, elevation—1,231 feet above mean sea-level, January and February, rainfall of area over 50 inches

(2) Breeding-places slowly running forests stream in a deep ravine fed by hill-foot seepages in the vicinity, completely covered by trees on both sides

Sunlight penetrated at places only for a short period, there were fallen leaves rotting everywhere and an absence of weeds and algæ in the stream which had a sandy zig-zag course with pockets and cul-de-sacs along the sides, the water being fairly cool and clear. Breeding was chiefly in the cul-de-sacs.

(3) Larval description. Small darkish brown coloured larva with the following characters —

- (a) Anterior clypeal hairs closely set and the internal ones more or less approximated, all of them slightly branched, with the internal ones branching from their middle
- (b) Posterior clypeal hairs are very small, branched from base and quite well apart
- (c) Antenna with a branched median hair and a basal hair of normal character
- (d) No filament to thoracic palmate, all abdominal palmates well developed

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# STUDIES ON THE ANTINEURITIC VITAMIN

## Part III.

### FURTHER EVIDENCE OF THE COMPOSITE NATURE OF B VITAMIN

BY

JOHN LEWIS ROSEDALE, D SC, F I C,

*From the Biochemical Department, King Edward VII College of Medicine,  
Singapore*

[Received for publication, March 4, 1929]

B VITAMIN has been observed by several workers to be at least of dual nature (Goldberger and associates 1926, Chick and Roscoe 1927, Sherman and Axtmayer 1927, Rosedale 1927). With the exception of Rosedale, who used pigeons as test animals, these authors have brought forward evidence that the complex vitamin is divided into an antineuritic factor, and a factor which cures or prevents symptoms of pellagra. The two factors have been termed B<sub>1</sub> and B<sub>2</sub> respectively by the English workers, and F and G by Sherman and Axtmayer.

These substances are both found in yeast and have been differentiated by autoclaving, which destroys the antineuritic factor B<sub>1</sub>. This factor may also be separated by adsorbing it upon norite charcoal or other suitable absorbent. Both factors appear to be essential to rats. Recent work by Hunt (1928a) shows that wheat and maize are richer in factor B<sub>1</sub> than in B<sub>2</sub>, and that B<sub>2</sub> is the limiting factor for growth. This has also been recognized by Chick and Roscoe (1928) who take as a standard for the assay of B<sub>2</sub>, that amount of substance which secures a weekly increase in the weight of their rats of 10 to 12 grammes, since upon purifying their caseinogen, they find the pellagrous symptoms more variable. Rosedale (1927) did not separate his factors by autoclaving, but by precipitating an extract of rice polishings with lead acetate. Small doses of either the filtrate or the precipitate would prevent the onset of polyneuritis in pigeons for long periods. The filtrate would also cure typical avian polyneuritis (head-retraction), but the precipitate would not do so even though concentrated to one-hundredth

of its bulk. The filtrate, therefore, appears to contain the factor  $B_1$ . When allowed to ferment, it fails to cure polyneuritis (Rosedale and Olivero, 1928). The nature of the factor contained in the precipitate portion is not clear from the preceding work, and the experiments reported below were designed to throw further light upon its properties.

## EXPERIMENTAL

*Controls*—Since Rosedale and Olivero (1928) have pointed out that on standing at laboratory temperature, the filtrate portion containing the antineuritic vitamin readily ferments, with loss of vitamin, it was necessary to make frequent extractions. Each batch of filtrate has been tested on cases of typical head retraction which has been cured by a dose of 5 c c given by the mouth. Batches not passing this test have been discarded. It has been observed that on ageing, rice polishings have not given a sufficiently strong extract, and it has become necessary once or twice to replace the stock of dry polishings with fresh material on this account.

## EXPERIMENTS

1 *Ration of raw white polished rice alone*—It has been found impossible to keep pigeons for more than 35 days on this ration. During the course of the work 55 pigeons have been placed on this diet, of which 36 have developed the typical head retraction with convulsions. These have been cured by the administration of 5 c c of the filtrate extract. The remaining 19 birds did not exhibit head retraction, but became more or less paralysed in limb, and could not be cured by the extract.

2 *Ration of cooked white polished rice*—When the rice was cooked, head retraction was not observed, although the 37 birds used for this experiment have all shown paralysis of limbs. These could not be cured by the extract. The observations of Grey (1928) prevent special emphasis being laid on the difference found after cooking the rice, but in view of the number of birds showing typical symptoms in experiment 1, the matter merits further investigation.

3 *Ration of white polished rice + 2 c c daily of extract*—Two c c of either the filtrate or the precipitate prevent the onset of polyneuritis for long periods. Birds were kept on each extract separately, but after 20 weeks the appetite began to fail, first in those receiving the precipitate, followed rapidly by those receiving the filtrate. Increasing the dose made but little difference in either case. Combining the extracts—that is giving daily 2 c c each of the precipitate and the filtrate extracts—revived the appetite.

4 *Ration of white polished rice + 5 per cent salt mixture + 5 c c orange juice*—The salt mixture was added to the rice and the orange juice was fed separately to each bird daily. The salt mixture was No. 185 of McCollum and Simmonds (1918). Its composition is— $\text{NaCl}$ , 0.173 gm,  $\text{MgSO}_4$ , 0.266 gm,  $\text{K}_2\text{HPO}_4$ , 0.954 gm,  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 0.347 gm,  $\text{CaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 0.540 gm,  $\text{Fe citrate}$ , 0.118 gm,  $\text{Ca lactate}$ , 1.30 gm.

Of 10 birds fed upon this diet, the head retraction was obtained in all birds within 40 days

5 *Ration No 4 + 2 c c daily of the filtrate extract*—This diet maintained 6 pigeons for over 7 months without depreciation in health or in appetite, and was then discontinued. It appears that this diet is similar in effect to that of rice and both extracts (experiment 3). The effect of the precipitate extract seemed therefore to be similar to that of the salt mixture, or the orange juice, or to a combination of the two

6 *Ration of white rice + 5 per cent salt mixture + 2 c c daily of the filtrate*—Four birds were maintained for 26 weeks. Subsequently, the loss of appetite noticed in experiment 3 was observed

7 *Ration of white rice + 5 c c orange juice + 2 c c filtrate daily*—Four birds used in this experiment were maintained in health and appetite throughout the course of the experiment—7 months. It appeared, therefore, that orange juice contained the factor which was also contained in the precipitate extract

During the course of the work Zilva (1927) published a paper in which he describes the precipitation of the anti-scorbutic factor C by lead acetate, which is similar to the method of treatment of rice polishings adopted in this laboratory. This procedure was, therefore, repeated both with orange juice and with pine-apple juice, and the previous experiment (No 7) repeated using each of these products separately. Pine-apple juice appeared equally as effective as orange juice and no signs of ill health were observed in either case

It was, therefore, thought desirable to test the anti-scorbutic potency of these substances on guinea-pigs. The basal diet was crushed oats, and a pair of guinea-pigs was used in each test. Four animals were given the basal diet only to act as control. These animals died within 30 days, exhibiting marked signs of scurvy

8 *Ration of oats + 10 c c orange juice extract*—The guinea-pigs on this ration survived the test period of 90 days without showing signs of scurvy

9 *Ration of oats + 10 c c 'pine-apple extract*—The guinea-pigs on this ration similarly survived the period of 90 days

10 *Ration of oats + 20 c c precipitate extract from rice polishings*—One guinea-pig died on the 19th day and the other on the 22nd day, both showing marked signs of scurvy

The connection between rice polishings and the fruit juices is not made clear by these experiments. Plimmer and Rosedale (1923) kept pigeons and other birds for long periods upon diets containing no C vitamin, and it was possible to rear a second generation from them. The birds of Plimmer and Rosedale, however, received oatmeal as the main bulk of their diet with the exception of two groups of chickens which were fed on white polished rice. The B vitamin supplement to the rice was dried yeast in one case, and yeast extract in the other. It is clear from their report that these authors found the dried yeast superior to the extract. More recently Hunt (1928b) has found that the residues of dried yeast after removing the B<sub>1</sub> and B<sub>2</sub> factors, contains yet a third substance which seems necessary to the optimum development of rats



In view of the trend of the foregoing experiments, it was of interest to attempt to ascertain the whereabouts of the anti-pellagra factor  $B_2$  in the rice polishings extracts. The experiments of Hunt (1928a) were not published at the time these experiments were undertaken, but the work of Chick and Roscoe (1927) had shown that  $B_2$  was not so abundant as the  $B_1$  factor in wheat embryo, so that it seemed possible that this might be a general characteristic of cereals. It has long been known that rats require the antineuritic factor  $B_1$ , which can be removed from these extracts by autoclaving, but without the use of some suitable absorbent there seemed no way of removing the factor  $B_2$  without destroying  $B_1$ . Chick and Roscoe had shown, however, that young rats failed to grow, and developed signs of sickness in 6 to 8 weeks unless supplied with the anti-pellagra factor, so that it appeared that if rats could be induced to survive this period without signs of ill health or lack of growth, it might be assumed that a certain amount of this factor was present. An experiment was carried out on 6 young rats of average weight, 40 grammes. They were placed on the diet of Chick and Roscoe (1927) purified casein 21 per cent, rice 63 per cent, coconut oil 11 per cent, salt mixture 5 per cent. Since it had been found in this laboratory that coconut oil, when exposed to the sun, will take the place of cod-liver oil, none of this latter oil was given. Within 4 weeks the rats had lost from 4 to 5 grammes in weight and had become feeble, so that this diet was suitable as a basal diet for the purpose of testing the extract. A batch of four rats (average weight 35 grammes) was placed upon this basal diet except that 5 per cent of the amount of rice was substituted by 5 per cent of the filtrate extract. Normal growth and health were maintained in these rats for the first ten weeks of experiment, so that it appeared that the anti-pellagra factor is contained in the filtrate and is not precipitated by lead acetate.

Subsequently, however, a loss in appetite and a slight loss in weight occurred. Two rats died in the 14th week and exhibited a dropsical or wet condition. At this time 5 c.c. of the precipitate portion was added to each 100 grammes of the diet in place of another 5 grammes of rice. One of the remaining rats died during the 18th week but showed no sign of dropsy, congestion of food in the intestine, however, was very marked. The fourth rat improved in health and continued to grow and the experiment was concluded at the end of the 22nd week. In none of these cases was it possible to observe any dermatitis.

These results seemed sufficiently interesting for repetition, and it was decided to include pigeons as well as rats in the test.

Six rats (average weight 40 grammes) were placed as before upon the basal diet together with 5 per cent filtrate extract. The first death occurred in the 13th week, followed by two deaths in the 15th week and a further death in the 16th week. Each rat showed signs of dropsy and of stasis. Five per cent of the precipitate was now added as in the last experiment and growth in the two remaining rats was resumed in the 20th week. The experiment was continued until the 39th week when the two rats were chloroformed. Post-mortem examination failed to reveal signs of dropsy or stasis.

Five pigeons were placed upon ration 3, white rice + 2 c c daily of the filtrate extract. During the 18th week scattering of food took place and two birds were found to be in a mopy condition. One of these died after three days in this condition showing dropsy and stasis at post-mortem. The other bird survived for another 10 days. This bird showed much stagnation of food in the intestines but not dropsy. Two c c of the precipitate portion were now given daily to each of the remaining birds. A third bird died in the 21st week showing marked stasis, but the other two appeared to regain their appetite and seemed in perfect health at the end of the 31st week when the experiment was discontinued.

In none of these cases could pellagrous symptoms be recognized either in the rats or pigeons.

## DISCUSSION

When an extract of rice polishings is completely precipitated by lead acetate, it appears that the original antineuritic vitamin discovered by Eijkman (1897) is not precipitated. This factor prevents or cures avian polyneuritis which bears resemblance to dry beri-beri in man. If the absence of the anti-pellagra factor,  $B_2$ , may be judged by the appearance of dermatitis or skin lesions, the foregoing experiments show that it is also not precipitated by lead acetate. A factor, however, has been precipitated by lead acetate whose absence in the diets of rats and pigeons has led to symptoms of stasis in the intestines. While there seems to be some indication that this factor may have some connection with wet beri-beri, this has not been proved. McCarrison (1928) makes a distinction between what he calls 'polyneuritis columbarum' and 'beri-beri columbarum,' but states that the head-retraction symptoms are common to both conditions. He considers that the pathological conditions of beri-beri columbarum are analogous to those of beri-beri in man, and that they are brought about by a shortage, though not a complete lack, of the vitamin. Plimmer and Rosedale (1926) pointed to a difference in symptoms between a total and a partial deficiency, but considered that while a total absence of vitamin resulted in typical head-retraction, partial deficiency led to a general debility and stasis. These experiments of McCarrison and of Plimmer and Rosedale were carried out upon the complete B vitamin complex. The above experiments tend to confirm the opinion of Plimmer and Rosedale, since the absence of the precipitated factor has, in the presence of the antineuritic vitamin, invariably produced stasis in the intestines of rats and pigeons. Orange juice and pineapple juice appear to contain the same substance as the precipitated factor, which, however, has been shown not to be the anti-scorbutic vitamin.

On the other hand, the more recent work of Chick and Roscoe (1928) indicates that typical pellagrous symptoms may not always be obtained, but that growth is the best indication of the presence of the  $B_2$  factor. These authors have, however, noticed that the appetite of their rats is not appreciably affected by the absence of the  $B_2$ . While falling off of appetite has been an invariable symptom in these experiments, it is possible that it may have been brought about by the onset of stasis. The work of Chick and Roscoe, and that of Hunt, shows

that cereals cannot be considered rich in the anti-pellagra factor McCarrison points out that prevention of polyneuritis in pigeons and beri-beri in man may be secured by the same means, by the replacement of some of the rice by atta and dhal It would appear, therefore, that the substance in atta and dhal is also present in rice polishings and in certain fruit juices, and that it differs from the antineuritic factor and from the anti-pellagra factor It would seem that a third member of the complex B vitamin exists, which may be important in the diet

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# THE ACTION OF CINCHONINE AND CINCHONIDINE ON THE VASO-MOTOR SYSTEM

BY

LIEUT -COL. R N CHOPRA, M A, M D (Cantab ), I M S,

B B DIKSHIT, M B, B S (Bom ), D P H (Cal ),

AND

K VENKATACHALAM PILLAI, L M S (Bom and Mad )

*(From the Department of Pharmacology, School of Tropical Medicine and Hygiene, Calcutta )*

[Received for publication, March 4, 1929]

## GENERAL AND PHYSIOLOGICAL CONSIDERATION

CINCHONA alkaloids, since their discovery, have been extensively used in therapeutics all over the world especially the tropics, but detailed pharmacological study of these alkaloids is still incomplete. Some alkaloids like quinine and quinidine have, of recent years, attracted the attention of pharmacologists and considerable experimental work has been done on them. The other two important alkaloids of cinchona bark—cinchonine and cinchonidine—have not been the subject of study of pharmacologists to the same extent as quinine and quinidine have been. Important textbooks on the subject refer to their action as only resembling that of quinine. The comparative action of cinchonidine and cinchonine on the heart was studied by Chopra, Dikshit and David (1928) who found that both depressed the heart muscle by direct action on the myocardium and increased the refractory and latent periods of the heart. It was also suggested that the vaso-motor centre was also responsible in producing the fall of blood-pressure, for after decerebration the fall was not so marked. The present research was undertaken to study the action of these alkaloids on the vaso-motor system.

In a study of the vaso-motor system as undertaken in this paper it is necessary to consider a few salient features of the physiological aspects of the circulation that have a direct bearing on the experimental observations recorded hereafter. The words 'rise' and 'fall' of blood-pressure after an injection are essentially relative and mainly depend upon the initial level of the mean arterial pressure

present before giving the injection. An animal with a very low mean arterial pressure will not show any fall of blood-pressure after an injection of a drug which ordinarily produces a distinct fall. On the other hand an animal with a very high initial blood-pressure level will show a more marked fall than one with a moderately high blood-pressure under identical conditions.

It is difficult to say which portion of the vaso-motor system is chiefly concerned in low blood-pressure which makes it less responsive to depressor stimuli. The pressor response is not abolished for stimulation of the vaso-motor terminals like that produced by an injection of adrenalin produces the usual effect. Stimulation of the ganglia by nicotine or of the centre by asphyxia produces the usual response though to a lesser degree and so the theory of paralysis of the vaso-motor mechanism cannot be held. It appears, however, that although no actual paralysis of the vaso-motor system exists, the tone of the arteries is very low and they are in a state of wide dilatation so that depressor stimuli do not evoke any further response from them. In our series of experiments we have found that a blood-pressure level of 40 mm of Hg or less will not respond by a fall of blood-pressure to ordinary doses of cinchonine and cinchonidine (2.5 mg per kilo of body-weight). All our observations were, therefore, made in animals whose initial level of blood-pressure was well above the lowest limit, and observations on animals whose blood-pressure was below 90 mm of Hg were not taken into consideration.

## EXPERIMENTAL

After the operation of section of the cervical cord a fall in blood-pressure occurs, due to interruption of tonic impulses that are constantly passing from the vaso-motor centre in the medulla. In our experiments, the section of the cord was done when the animal was well under chloralose and this anaesthetic was used in all our experiments to obviate any error due to difference in the effects of the anaesthetics used. After about an hour following the operation, the blood-pressure becomes quite steady and constant and retains a fairly high level to allow the study of depressor responses.

In measuring the fall, the calculations were made by taking the percentage of fall from the initial level of mean arterial pressure. The blood-pressure was recorded by a mercury manometer, attached to the right common carotid artery. Although this is not the ideal method for registering changes of blood-pressure, it was selected for the convenience of its use and the ease with which changes in blood-pressure are measured. All our experiments were conducted on cats weighing between 2 and 3 kilos. The anaesthetic selected was chloralose as it gives a very good initial level of blood-pressure. The alkaloids used were prepared by Merk and the base was dissolved in the minimum quantity of hydrochloric acid. The injections given were calculated in terms of the base. The dose in all animals was 2.5 mg of the base per kilo of body-weight. In perfusion experiments, the concentrations varied from 1—20,000 to 1—200,000.

Graph I, figs *a* and *b*, shows the effect of injections of cinchonine and cinchonidine given intravenously. There is a well marked and persistent fall of blood-pressure, the fall is fairly sudden, the blood-pressure reaching its lowest level in less than half a minute, the pressure recovers gradually but never attains its original level. The degree of fall varies according to the tone of the arterial system before the injection is given, the average fall, under ordinary conditions, is about 29 per cent (average of six observations). The spleen shows a marked and prolonged increase in its volume. Sometimes a slight fall in the spleen volume is observed and this is followed immediately by an even greater rise. The cause of the slight initial fall may be depression of the heart, for the drug reaches the heart before it goes to the arterioles. It may be observed, however, that a slight initial constriction of the blood-vessels occurs when organs are perfused with solutions of these alkaloids before a marked dilator effect is manifested. It is possible that this may be responsible for the initial fall of the spleen volume. The marked fall of systemic blood-pressure with the simultaneous increase in the volume of the splanchnic area generally and spleen particularly indicates that this may be an important factor in producing a fall of systemic blood-pressure, since the limb volume records taken simultaneously do not show any change. The depression of the heart may also contribute to some extent, but this, as will be shown subsequently, is not the chief factor.

Along with the rise of spleen volume, the rhythmic movements of the spleen are also increased, if they are absent before the administration of the alkaloids they soon start after such administration. Whether the increased mobility is due to any specific action on the part of the alkaloids or whether it is merely due to the distension of the organ is difficult to say. It seems more probable that the increase in the rhythmic movements of the spleen is chiefly due to distension of the organ and not to any specific action of these substances. Injections of other drugs such as organic antimonials have also been shown by Chopra (1927) to increase the movements of the spleen. Similarly also large quantities of normal saline increase or initiate the rhythmic movements of the spleen. Whatever might be the cause, the fact that rhythmic movements of the spleen are increased appears to be of some importance so far as the curative action is concerned, as the parasites located in that organ are thrown out into the circulation to be acted upon by the drug. This has been shown by Chopra and Das Gupta (1928) to be the case with organic compounds of antimony when administered in kala-azar patients.

The marked increase in the spleen volume suggests that there is dilatation of the vessels of the splanchnic area. In order to see whether other abdominal viscera showed similar increase, the volume of the intestines was recorded, it was found that it definitely increased after injections of these alkaloids in ordinary doses. Perfusion of the superior mesenteric artery, as will be shown subsequently, produced a marked dilatation of the vessels and the venous outflow from the intestinal area was increased. We are, therefore, justified in concluding that the alkaloids produce a marked dilatation of the vessels of the splanchnic area. From

these data it is evident that the fall in blood-pressure produced by cinchonine and cinchonidine is in great part due to their dilator action on the splanchnic blood-vessels. In order to elucidate the mechanism of this dilatation, we studied the different components of the vaso-motor system to determine which of them was chiefly affected.

The vaso-motor centre was put out of action by severing the cervical cord of an animal under chloralose, at the level of the 7th cervical. The blood-pressure after this operation was lower than the normal blood-pressure level but was at a sufficiently high level to show the depressant action of the drugs injected. After waiting for a couple of hours the blood-pressure and the spleen volume were recorded and the injections of the alkaloids were given. The qualitative effects observed on both were those usually obtained but quantitatively there was a marked difference. The average fall of blood-pressure in the case of cinchonine was about 12 per cent and in the case of cinchonidine it was about 7 per cent. The fall in animals under chloralose with the centre intact was, as already observed, 29 per cent. The rise in spleen volume after severance of the cervical cord was comparatively small and sometimes absent, no increase in the rhythmic movements of the spleen was observed. It will be seen from these observations that depression of the vaso-motor centre is one of the causes of the dilatation of the splanchnic vessels and lowering of the blood-pressure.

This method of studying the vaso-motor centre is open to objection. It may be argued that interruption of tonic impulses from the centre will produce a relaxation of the tone of the arterioles which, therefore, will not respond equally well to depressor stimuli. To study the vaso-motor centre more accurately the technique developed by Sollmann and Pilcher (1910) was adopted. The limb, or one of the abdominal organs (kidneys), was perfused with warm oxygenated Locke's solution to which defibrinated blood was added, while the nervous connections of the preparation were not disturbed. The outflow from the vein of the perfused limb was calculated by counting the drops emerging from the vein and recording them on a moving drum. Graph I, figs *c* and *d*, shows the effect of an intravenous injection of cinchonine and cinchonidine in such experiments. It will be seen from the graphs that the rate of flow of perfusate is increased after an injection of the alkaloids showing that a dilatation of the vessels is caused by depression of the centre. The alkaloids in these experiments could only act through the vaso-motor centre since they were not reaching the perfused limb.

It has been observed before that after severance of the spinal cord, the fall of blood-pressure and the rise of the spleen volume observed after injections of cinchonine or cinchonidine were not so marked as before the section. To determine the part played by the vaso-motor nerve-endings, sufficiently large doses of ergotoxin were given to paralyse them. The injections of the alkaloids were then repeated, the blood-pressure and spleen volume being recorded as before. It was observed that the degree of fall of blood-pressure was not so great as that observed after severance of the spinal cord. There was no rise in the spleen volume and the rhythmic movements of this organ were markedly decreased or

very often abolished altogether (Graph II, fig *a*) In the case of cinchonine the average fall of blood-pressure was 7 per cent, while with cinchonidine it was 4 per cent The following table gives the variations in the fall of blood-pressure observed in animal under chloralose only, after section of the cord and after paralysis of the vaso-motor terminals with ergotoxin —

	PERCENTAGE OF FALL OF BLOOD-PRESSURE	
	Cinchonine (Per cent)	Cinchonidine (Per cent)
Animal under chloralose only	29	29
After division of the cord in the cervical region	12	7
After sufficient ergotoxin to paralyse vaso-motor nerves	7	4

A perusal of the above results shows that depression of the vaso-motor centre as well as the terminals of the vaso-motor nerves are responsible for producing dilatation of the blood-vessels, after injections of the alkaloids cinchonine and cinchonidine

The limb or the kidney of a cat were perfused with warm oxygenated Locke's solution to which defibrinated blood was added Varying quantities of the alkaloids were added to the canula leading to the vessels through which perfusion was being done so as to attain concentrations varying between 1 in 75,000 to 1 in 50,000 Graph II, fig *b*, shows the acceleration of flow observed after an injection of cinchonine showing that a well marked vaso-dilatation is produced The vaso-dilatation, however, is preceded by a slight constriction probably produced by the initial momentary stimulation of the blood-vessels Cinchonidine produces exactly similar results

To determine the action of the alkaloids purely on the arterial system, exclusive of the effects on the capillaries and the veins, we perfused the superior mesenteric artery with warm oxygenated Locke's solution containing defibrinated blood The perfusate was put in a mercury bulb with a tightly fitting rubber cork at the top through which a glass-tube was passed This arrangement maintains the pressure of the perfusate at a constant level and the entry of the air bubbles serves to oxygenate the fluid and measure the inflow A canula was introduced in the superior mesenteric artery and the intestinal attachment of the mesentery was cut throughout its entire length The perfusate introduced through the canula, therefore, finds its way to all the minute branches of the superior mesenteric artery and comes out through the cut ends of the small arterioles Introduction of a drug in the canula will thus act only on the arterial system Injections of cinchonine and cinchonidine only in concentrations of 1 in



50,000 and more produce dilatation of the arterioles large enough to be measured by the acceleration of the rate of entry of air bubbles in the perfusate. In one of the experiments, the time required for 10 bubbles of air to enter was 13 seconds before giving the injection and after an injection of cinchonidine it was 9 seconds, showing the acceleration of flow after cinchonidine. Similarly after cinchonine the flow was accelerated. With lower concentrations the effects are not so apparent. These experiments go to show that both cinchonine and cinchonidine produce dilatation of the arterioles and thus produce a fall in blood-pressure. This acceleration of flow is not observed after paralysing the vaso-motor terminals by ergotoxin, showing that the dilator action is probably brought about by the direct depression of the vaso-motor nerve-endings.

Further proof of this depressant action of the two alkaloids on the vaso-motor nerve-endings was sought by injecting large quantities into animals and studying the pressor responses of exactly the same quantity of adrenalin before and after such an injection. Graph II, figs *c* and *d*, show the effect of 1-40th c.c. of adrenalin before and after giving 34 mg. of cinchonidine. It will be observed that the pressor response of adrenalin is considerably less after large doses of cinchonine and cinchonidine showing that these two drugs depress that mechanism of the vaso-motor system which is stimulated by adrenalin, i.e., the sympathetic nerve endings. Direct depression of the arterial muscle by the alkaloids may also play a part in reducing the pressor response, but in view of the experiments already cited we are inclined to believe that the chief factor is nervous and not muscular. Rings of aorta taken from a freshly killed rabbit suspended in Fleish's solution containing defibrinated blood show only a slight diminution of tone in such concentration as 1—50,000, which are not likely to be attained in the tissues.

It will be seen from the experimental data given above that both cinchonine and cinchonidine produce a fall of blood-pressure mainly by (1) dilatation of the arterioles due to the depression of the vaso-motor centre, and (2) the depression of the vaso-motor nerve-endings. All the cinchona alkaloids are known to be protoplasmic poisons and so may act directly on the musculature of blood-vessels and depress it. Perfusion experiments after ergotoxin, however, go to show that this depressant action on the muscle fibres of the blood-vessels does not come into action even in such high concentrations as 1 in 10,000 and, therefore, in all probability does not play any part when ordinary doses are given.

A study of the vaso-motor system will be incomplete without studying the veins and the capillaries. We observed the effect of these alkaloids on the capillaries by direct observation under the microscope in the omentum of frogs and cats, but the disadvantages of such a procedure are obvious. Injection of the alkaloids into the circulation depresses the heart and slows the movements of the erythrocytes through the capillaries giving an impression that the capillaries are dilated. Small changes in the calibre of the capillaries are difficult to observe under the microscope. Application of different concentrations of the alkaloids even so high as 1 in 20,000 directly to the capillaries did not show any appreciable change in the diameter of these vessels.

The record of venous pressure likewise presented many difficulties. Ligature of a vein and taking the pressure by a canula directed against the blood-stream is not the correct way of recording the venous pressure as the occlusion of the vessel gradually increases its pressure till an equilibrium in the vein, the capillaries and the nearest arterioles is established. We, therefore, recorded the venous pressure in the following way —

A canula was introduced in the femoral vein directed towards the heart and was connected to one limb of a U tube, the other limb being connected with a recording tambour. Before establishing the connection, however, the U tube was filled with weakly citrated saline and the pressure allowed to adjust itself. Any change in the venous pressure would thus be recorded by the saline going into the vein or the blood entering the canula. Introduction of the canula in the femoral vein, lower down in the thigh, would not only record the pressure in the veins above the seat of canula in that limb but would also give an indication of the venous pressure on the opposite side. The rise or fall of venous pressure was recorded on a moving drum. In our series of experiments we observed that cinchonidine and cinchonine given in ordinary doses do not produce any appreciable change in the venous pressure.

The marked dilatation of the arterioles produced by these alkaloids might suggest that the venous pressure would rise. It is difficult to explain the absence of any change in the venous pressure although the arterial pressure suffers such marked changes. Most probably the 'capacity factor' as pointed out by Bayliss, prevents any change in the venous pressure to be appreciated. The wide dilatation of the vessels locates a good volume of the blood in their lumen so that although the arterial blood-pressure falls considerably the venous pressure remains unaltered.

The changes in the venous flow are usually measured by recording drops of blood emerging from the cut end of a vein. This procedure involves a certain amount of bleeding which undoubtedly vitiates the results. Experiments were, therefore, devised which necessitated comparatively slight bleeding from the veins, necessary compensation being made by introducing warm normal saline into the circulation. In studying the venous outflow of a peripheral area (e.g., a limb), the rate of flow of blood through a tributary of the femoral vein coming from the adductor muscles was measured. The vein itself is too small to allow introduction of a canula and the difficulties of clotting are great. The femoral vein was, therefore, ligatured above and below the junction of the said tributary and two canulae were introduced both directed towards the heart, one a little below and the other just above the tributary. Through the distal canula citrated saline was run at a constant pressure at a fixed rate and the outlet from the proximal canula was measured. The outflow consisted of the citrated saline and the blood from the vein coming from the adductors. As the saline flow was constant any change in the rate of flow was due to increased flow of blood through the vein. We found by this method that after usual doses of cinchonine and cinchonidine there was no appreciable change in the venous flow of the peripheral area. Similar experiments conducted on the venous flow of the splanchnic area (the intestines),

GRAPH I

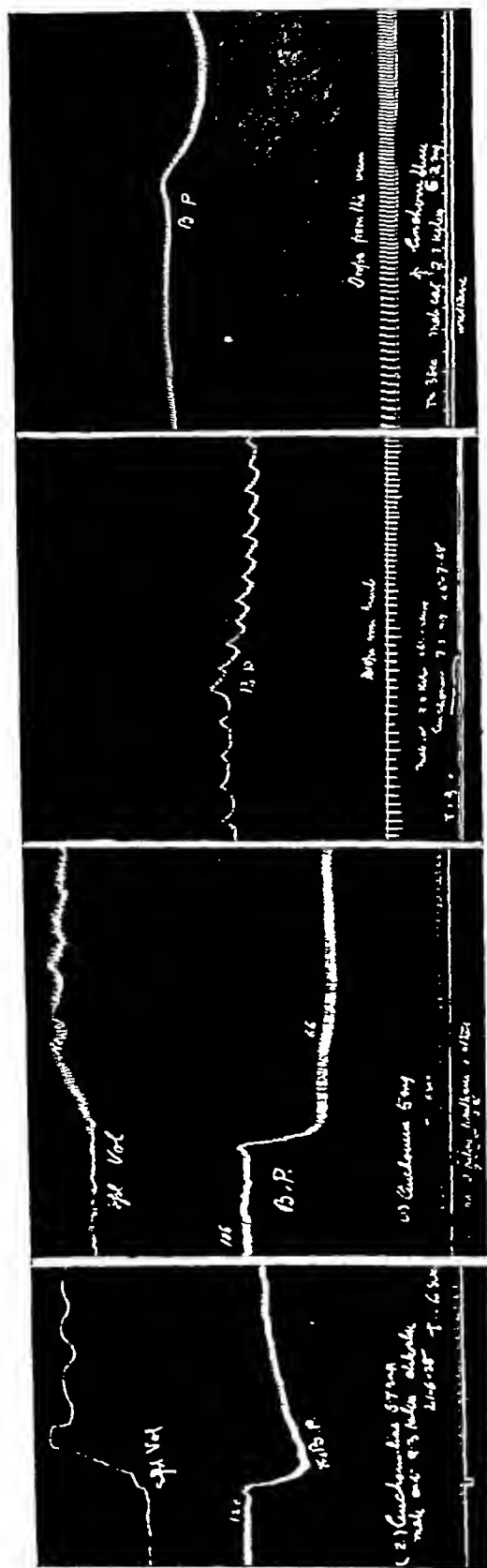


Fig a and b—Upper tracing spleen volume lower tracing blood pressure. Note the fall of blood pressure produced by encephalitic and encephalitic respectively. Volume of the spleen is increased and the rhythmic contractions stimulated.

Fig c and d—Upper tracing blood-pressure lower tracing drops from perfused limb. The depressing effect of the alkaloids on the visomotor centre is shown by the depression of the blood-vessels of the perfused limb (where nervous connections are intact and vascular connections are severed so that the alkaloids are acting through the centre only). Note the flow is accelerated showing marked dilatation.

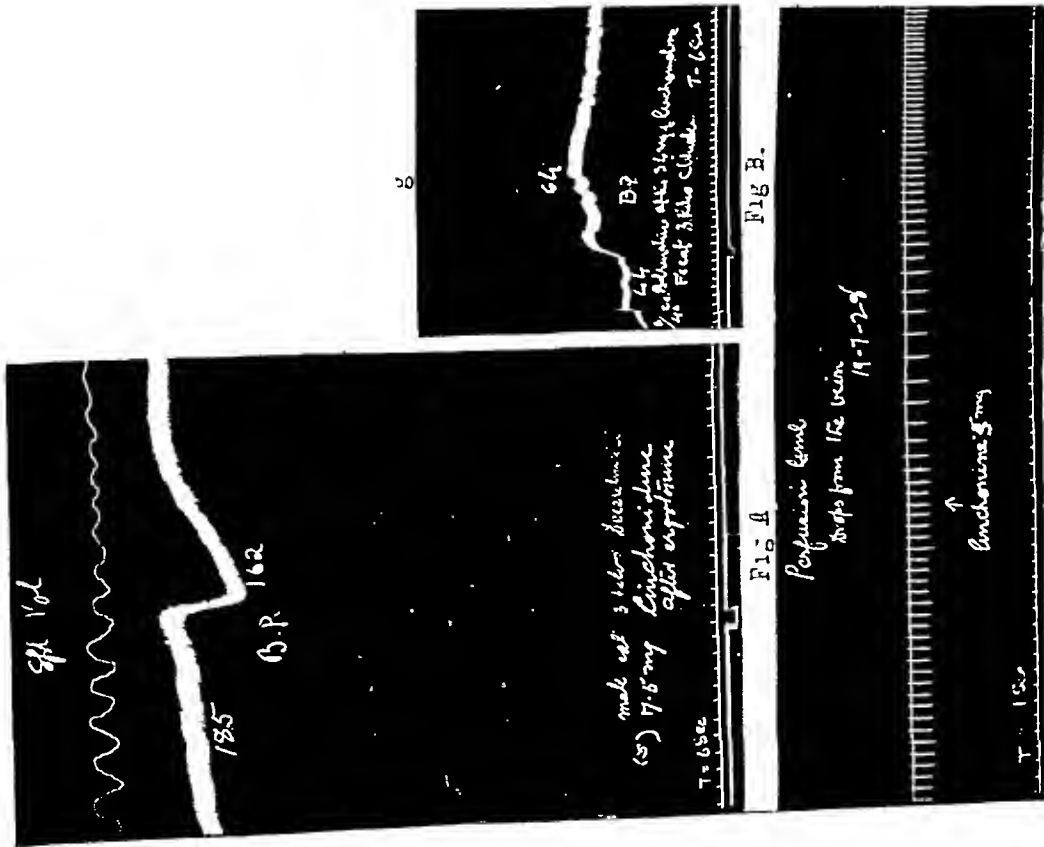
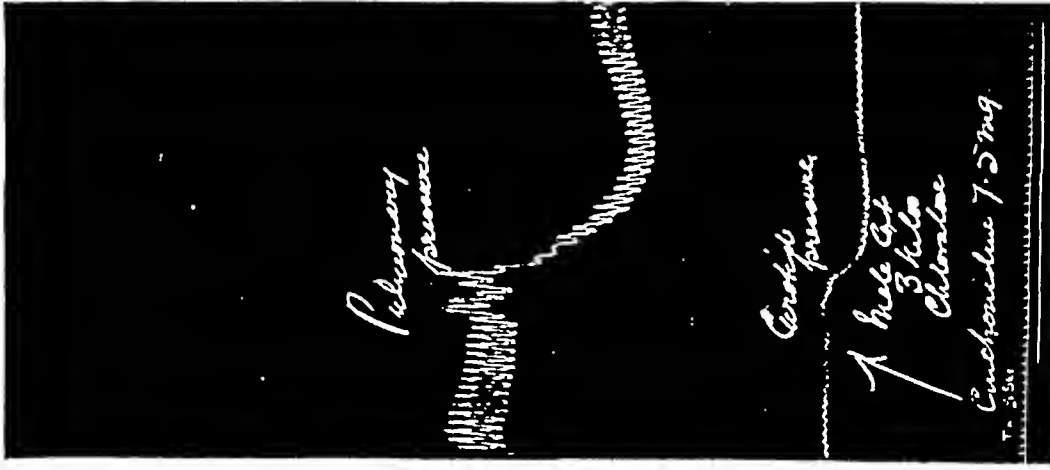


Fig a—Upper tracing spleen volume, lower tracing blood-pressure. Note the effect of cinchonine after paralysis of vaso-motor nerve-endings with ergotamine. The fall of blood-pressure is not marked. The rise in volume of spleen is absent and rhythmic movements diminished. Fig b—Perturbation of a limb with 1 in 50,000 solution of cinchonine. Note dilatation of blood-vessels preceded by slight contraction as shown by the increase and decrease of drops coming out from the vein. Fig c—Shows the effect of 1/40th c.c. of adrenaline before cinchonine. Fig d—Shows the effect of 1/40th c.c. of adrenaline after 34 mg. of cinchonine.



Figs e and f—Show the effect of cinchonine and cinchonidine respectively on the pulmonary blood-pressure. Upper tracing pulmonary pressure, lower tracing carotid pressure. Note that cinchonine shows a rise in pulmonary pressure while cinchonidine shows a fall.

however, showed that venous outflow from the splanchnic veins is increased, after cinchonine and cinchonidine

In all the above experiments only one observation was taken in one animal and the changes recorded in as little time as possible to obviate any error due to loss of blood. The loss in all cases was very small as only a small vein was allowed to bleed. The bleeding by itself never produced any change in the systemic blood-pressure as shown by the carotid tracings. The increase of flow of venous blood in the splanchnic area and the absence of any change in the peripheral area (e.g., limb) show that the dilator action is chiefly manifested in the splanchnic area and not in the peripheral

Changes in the pulmonary circulation were studied by recording the pressure in the pulmonary artery. This is the only instance where the action of the two alkaloids differed. Graph II, fig *e*, shows the effect of an injection of cinchonine and Graph II, fig *f*, that of cinchonidine on the pulmonary pressure. While cinchonidine produces a distinct fall in the pulmonary pressure, cinchonine raises it, sometimes very markedly. This difference is probably due to the difference of action of these two drugs on the heart as pointed out by Chopra and others (1928). Cinchonine stimulates the auricles while cinchonidine depresses it and this stimulation may account for the increase in the pulmonary pressure in the case of cinchonine.

### SUMMARY

The experiments given above indicate that the fall of blood-pressure after ordinary doses of cinchonine and cinchonidine is produced by the depression of (1) the vaso-motor centre, and (2) vaso-motor nerve-endings. The effect is mainly manifested in the splanchnic area as shown by the rise in the spleen volume and increased venous outflow of the splanchnic veins. These alkaloids when perfused into a limb dilate its arterioles, but its volume and the outflow from its veins are not markedly affected. This difference in action is perhaps due to such compensation effects as are brought about reflexly in the Loven reflex. The wide dilatation of the splanchnic blood-vessels will constrict the peripheral blood-vessels to some extent so that the dilator effect of the drugs on the peripheral circulation is compensated for. Thus although perfusion experiments show the dilator response markedly, the same results are not obtained in the intact animal, where the volume of the limb or the venous outflow of the limb is recorded.

### CONCLUSIONS

(1) The alkaloids cinchonine and cinchonidine, given in doses of 2.5 mg per kilo body-weight, produce a fall of blood-pressure and dilatation of the blood-vessels of the splanchnic area.

(2) This dilatation is due to depression of (a) the vaso-motor centre, and (b) of the vaso-motor nerve-endings. Direct depression of the muscle fibres of the vessel is not an important factor.

(3) Perfusion experiments show that the peripheral blood-vessels such as those of the limbs are also dilated

(4) Pulmonary pressure is increased by cinchonine and lowered by cinchonidine

(5) Venous outflow in the splanchnic area is increased while in peripheral area (limbs) it remains unaltered

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# A PRELIMINARY NOTE ON CERTAIN FACTORS INFLUENCING BACTERIAL GROWTH

BY

LIEUT M L AHUJA, M D, CH B, I M S,  
*Central Research Institute, Kasauli*

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CERTAIN aspects of this subject, e g, the effects of variations in the nature of soil, time and temperature of incubation, etc, on the luxuriance of bacterial growth, have been dealt with in detail by Norris (*Ind Jour Med Res*, Vol VII, p 536)

The experiments described in this communication were designed to determine the effects of factors, other than the nature of soil, which are responsible for variations—sometimes very marked indeed—in the total bacterial yield

By growing organisms under exactly identical conditions and altering one variable only at a time, results have been obtained which are interesting enough to warrant publication

## *Relation of amount of seed to Bacterial Yield*

Douglas agar plates of 20 sq cm area were seeded with *B typhosus*—amount of seed varying from 100 organisms to 15,000 million organisms. Seed was suspended in 0.4 cc of normal saline. Bacterial yield was determined after 24 hours' incubation at 37°C

There is a steady increase in yield up to 30 millions, beyond this limit increasing the amount of seed makes no difference to the total yield. Fifteen thousand millions yield exactly the same growth as 30 million in a soil area of 20 sq cm, thickness of medium being constant

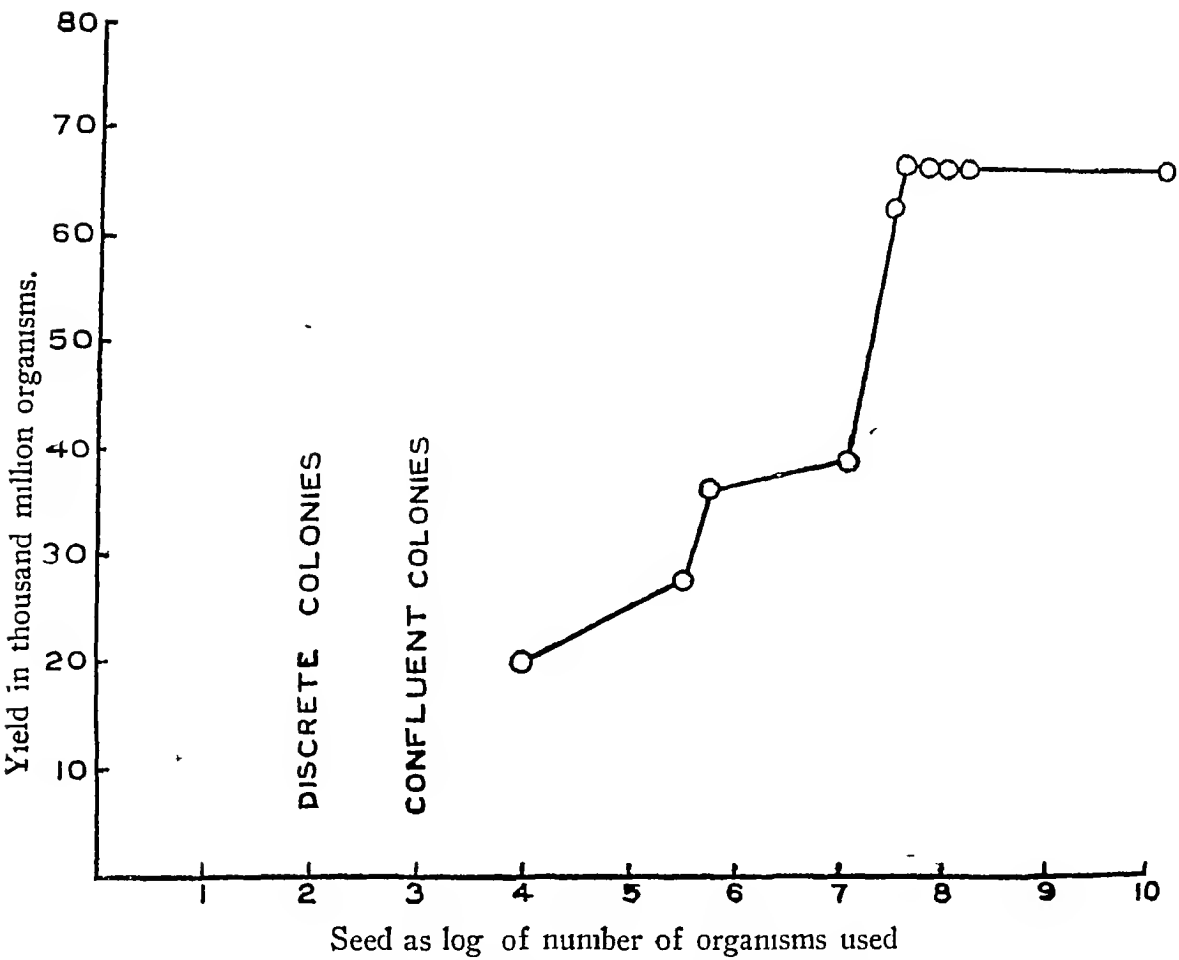
Provided the quantity of seed for a given soil area is not below a certain limit, the total yield resulting from increasing the amount of seed for that area remains constant. No beneficial effect is, therefore, obtained by increasing the seed beyond certain limits when the soil area is kept the same

TABLE I

See Chart 1

Seed (Number of organisms )	Yield (Number of organisms )
100	Discrete colonies
1,000	Confluent colonies
10,000	20 thousand millions
500 thousand	29 " "
1 million	37 " "
10 millions	40 " "
20 " "	64 " "
30 " "	69 " "
40 " "	69 " "
50 " "	69 " "
100 " "	69 " "
1,000 " "	69 " "
15,000 " "	69 " "

CHART 1.



*Relation of thickness of soil to Bacterial Yield*

By keeping the soil area constant and increasing the thickness of soil a marked increase results in the total yield, as is evident from Chart 2 0.2 cm

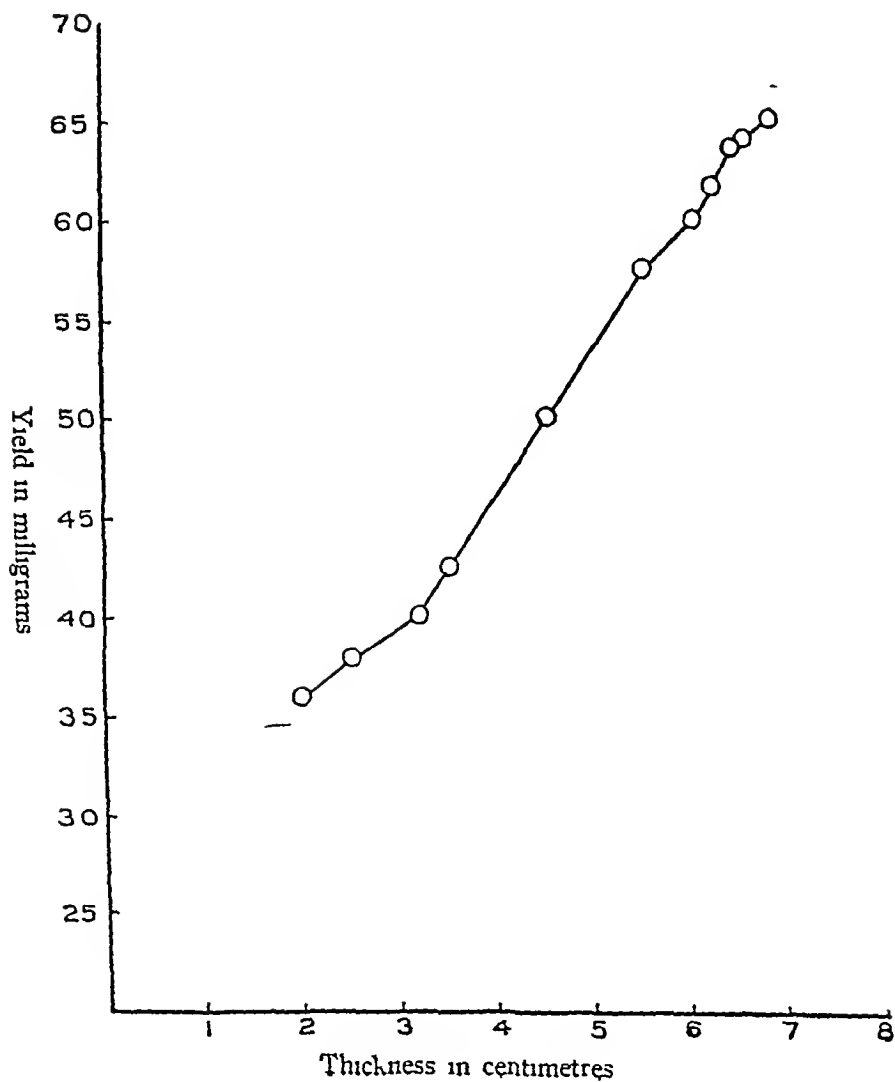


TABLE II  
Soil area = 26.4 sq cm

See Chart 2

Medium in c c	Thickness in cm	Yield in mg
10	0.2	36
12	0.25	38
14	0.32	40
15	0.35	42.7
17	0.45	50
19	0.55	57.5
20	0.6	60
22	0.62	61.8
24	0.64	63.5
25	0.65	64
27	0.68	65

CHART 2



thickness of soil gives a total yield of 36 mg after 24 hours' incubation at 37°C. If the thickness is increased to 0.6 cm, the total yield increases to 60 mg—an increment of 24 mg in a soil area of 26.4 sq cm.

This increase in yield, however, can be obtained only up to a certain limit, beyond that limit thickness of soil has no effect on increasing the yield, provided of course, soil area is kept constant.

TABLE III

*See Chart 3*

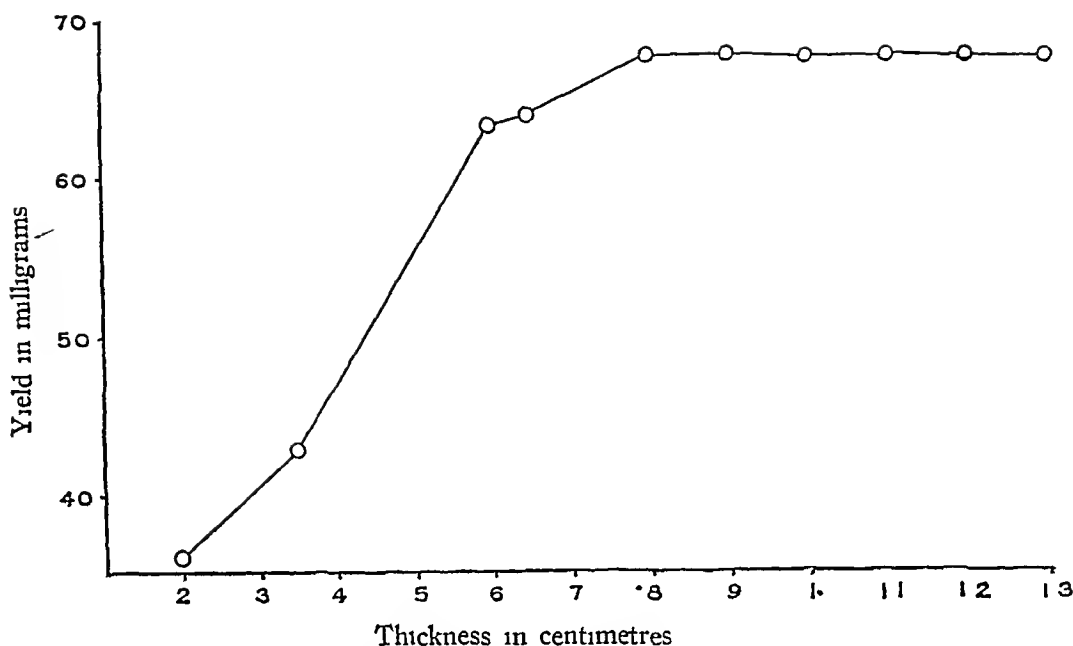
Medium in c.c.	Thickness in cm	Yield in mg
10	0.2	36
15	0.35	42.7
20	0.6	63
25	0.65	63.9
30	0.8	67.5
32.5	0.85	67.5
35	0.9	67.5
37.5	1.0	67.5
40	1.02	67.5
42.5	1.05	67.5
45	1.1	67.5
50	1.2	67.5
55	1.3	67.5
57.5	1.5	67.5

Although increase in yield can be obtained by increasing the depth of medium, it must be realized that the total quantity of medium employed is also considerably increased in order to obtain the desired increase in thickness. The question arises whether it is economical to take advantage of this thickness factor in increasing the growth.

The quantity of medium required to produce a thickness of 0.2 cm in 26.4 sq cm soil area is 10 c.c., the amount required to produce a depth of 0.6 cm is 20 c.c., i.e., twice as much. Now if the yields resulting from these different quantities of the same medium be compared, it is evident that

Ten c.c. of agar with a depth of 0.2 cm yield 36 mg of bacterial growth, while 20 c.c. of agar with a depth of 0.6 cm yield 63 mg of the same growth, i.e., less than double.

CHART 3



Increase in yield can be obtained by increasing the depth of soil but it is not an economical factor to take advantage of

*Relation of area of soil to Bacterial Yield*

Twenty c.c of agar were spread over an area of 20, 27.5, 30, 37, 40 and 45 sq cm. Each plate was seeded with the same amount of seed and the yield determined after 24 hours' incubation at 37°C

TABLE IV

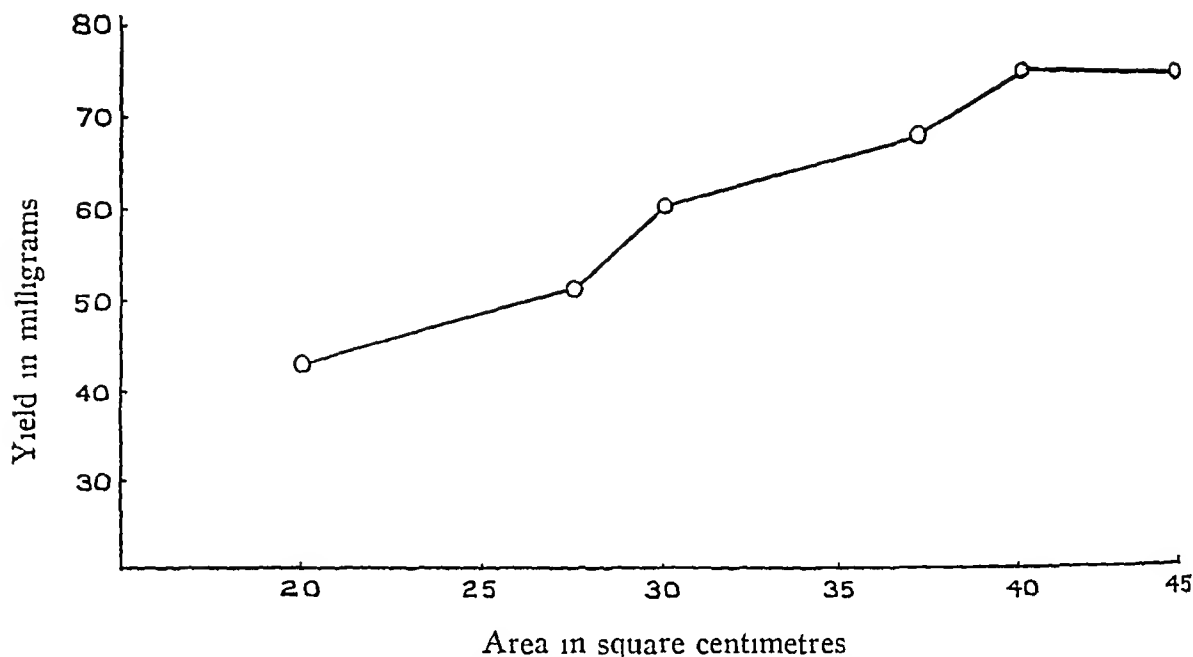
See Chart 4

Agar	Area	Yield
20 c.c	20 sq cm	43 mg
20 "	27.5 "	51.6 "
20 "	30 "	60.2 "
20 "	37 "	68.8 "
20 "	40 "	75 "
20 "	45 "	75 "

The quantity of medium employed in both cases being the same, the yield from 20 sq cm area is 43 mg whereas it is 75 mg from an area of 40 sq cm

Bacterial yield is considerably more when a given quantity of agar is spread over a bigger soil surface. This is also true up to a certain limit, for if the

CHART 4



area is increased too much, continuous film of growth gives place to confluent and discrete colonies and the whole of the soil area is thereby not utilized by the organisms employed as seed

*Relation of amount of menstruum to the total Bacterial Yield*

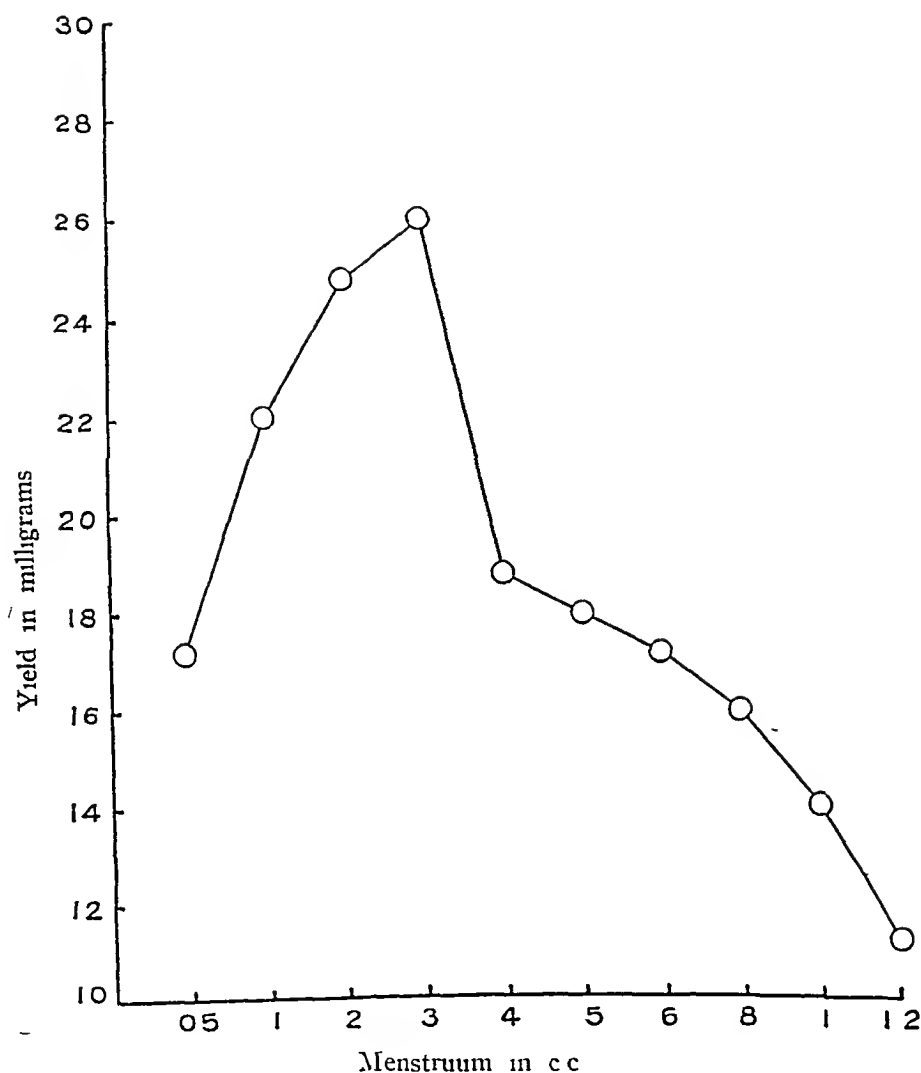
Douglas agar plates of the same soil area and same thickness were seeded with the same amount of seed—more than the optimum—suspended in various amounts of normal saline. Yield was determined after 24 hours' incubation at 37°C

TABLE V

See Chart 5

Menstruum	Yield
0.05 cc	17.2 mg
0.1 "	22.0 "
0.2 "	24.8 "
0.3 "	26.0 "
0.4 "	18.8 "
0.5 "	18.0 "
0.6 "	17.2 "
0.8 "	16.0 "
1.0 "	14.0 "
1.2 "	11.2 "

CHART 5



As is evident from the accompanying table and Chart 5, bacterial yield is influenced to a very marked degree, by the amount of fluid in which the seed is suspended. There is a steady rise in the total yield up to the use of 0.3 c.c. of saline but beyond this limit the yield becomes less and less as the amount of saline increases. 0.3 c.c. of saline may, therefore, be taken as the optimum amount of menstruum which gives the maximum growth in a soil area of 20.4 sq. cm.

As a matter of fact 0.3 c.c. of saline is just enough to cover 20.4 sq. cm. of soil area comfortably and no more. 0.4 and 0.5 c.c. or over leaves the soil area more or less drenched owing to excess of fluid, and the resulting yield

is consequently not a continuous film of growth, but a moist unseeded looking area in some parts of the plate and a fairly uniform growth in others

If the fluid is in excess, not only is the soil surface not utilized completely but the actual seed is wasted, owing to its being in suspension in that excess. Consequently owing to loss of seeding surface and also the seed, the yield is poor.

To get the maximum effect one must pay as much attention to the menstruum as to the amount of seed.

Similar experiments have been carried out making use of agar lined roll bottles with practically identical results. Each bottle was lined with 100 cc of casein Douglas agar, seeded with the same quantity of seed and incubated for 24 hours at 37°C. Bacterial yield was estimated by Brown's opacity method.

TABLE VI

Seed	Saline	Bacterial Yield
1 cc		25.6 mg
1 "	1 cc	33.6 "
1 "	2 "	26.4 "
1 "	3 "	21.6 "
1 "	4 "	20.4 "
1 "	5 "	16.8 "
1 "	6 "	20 "
1 "	7 "	18.0 "
1 "	8 "	18 "
1 "	9 "	17.2 "

## SUMMARY

Influence of certain factors, e.g., amount of seed, depth and area of soil, etc., on the total bacterial yield has been investigated.

# A PRELIMINARY NOTE ON THE ANTIGENIC VALUE OF A BACTERIAL VACCINE

BY

LIEUT M L AHUJA, M D, Ch B, I M S,  
*Central Research Institute, Kasauli*

[Received for publication, April 11, 1929]

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- (b) Agglutinable emulsion used for the estimation of 'heat labile' and 'heat stable' agglutinin titre
- (c) Technique of agglutination
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- (e) Experimental animals

AGGLUTINATION TESTS WITH RABBIT SERA (*L1pts I—VI*)

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IMMUNITY EXPERIMENT (*E1pt VIII*)

ANALYSIS OF RESULTS

## INTRODUCTION

During typhoid infection in man the immunity response shows a fairly close parallelism to the appearance and content of typhoid agglutinins in the patient's serum. The works of Weil and Felix (1920), Burnette (1924), Bruce White (1926) and several others in recent years, show that two kinds of antigens are contained in the organism, the labilotropic or 'heat labile' and the stabilotropic or 'heat stable'. These are demonstrable as large flaking or 'floccular,' and small flaking or 'granular' agglutinins respectively, and are probably associated with the rough and smooth variants of the organism. This subject of bacterial variation is of vital importance in the manufacture of vaccines, particularly so in the case of Army T A B vaccine, where each one of the three members is supposed to have at least two, and possibly four, variants, each variant probably possessing a markedly different antigenic and immunologic value than the other

In view of the opinion maintained by Felix (1924), that it is only the heat stable agglutinogens of the smooth variant which are responsible for protection against typhoid, one naturally feels chary—provided the contention be true—in sending out a prophylactic vaccine, which does not give rise to such heat stable agglutinins in the sera of immunized subjects

The experiments described in this article were designed to determine (a) the antigenic and immunizing value of the typhoid portion of the Army TAB vaccine and (b) the influence of variation in nutritional media on its antigenic response

Different workers lay stress on different types of anti-bodies as being responsible for defensive mechanism. Some attribute it to the opsonins, others to agglutinins and hæmolysins, and others, again, to complement deviating bodies. The actual part played by agglutinins in a serum, as defensive factors, is still a very moot point nevertheless such production may be accepted as the index of antigenic properties of a vaccine

In the present investigation antigenic value has been measured by agglutino-genic response only.

#### CULTURAL DETAILS

##### (a) *Organisms used*

1 *B. typhosus* 'smooth' (Rawlings), the same as is actually used for the production of the typhoid portion of TAB vaccine in the Central Research Institute, Kasauli

2 *B. typhosus* 'smooth' (Mrs S)

3 *B. typhosus* 'rough' (Mrs S)

(b) *Agglutinable emulsions*—The emulsions used for estimating the 'floccular,' or heat labile, content of immune sera were prepared according to Oxford University method, i.e., by growing the strain in broth for 24 hours, killing it by 0.1 per cent formalin and leaving it in a cold chamber for about a week, shaking it about three or four times a day in the intervals

'Granular' content of the immune sera was estimated by using alcoholized suspensions of a strain, sensitive enough to demonstrate heat stable agglutinins. A strain of *B. typhosus*, called 8 'o,' was kindly lent to me by Major R. F. Bridges, Commanding Enteric Convalescent Dépôt. This was found to be the most sensitive of all the available strains and was consequently employed in the preparation of alcoholized emulsions which were prepared as follows—

An 18 hours' growth of *B. typhosus* on Douglas agar slopes was mixed with 20 c.c. of absolute alcohol in a vaccine bottle containing sterile beads. After a couple of hours shaking in a mechanical shaker, the bottle was left at room temperature for 24 hours when the supernatant alcohol was pipetted off and the rest allowed to evaporate in the incubator at 37°C. Sterile doubly distilled water was then added to the dried organism and the solution standardized to contain 1,300 million organisms per c.c. according to Brown's opacity method

(c) *Technique*—Dreyer's technique of agglutination has been employed throughout the study







(d) *Preparation of vaccines*—Killed and carbolized cultures have been employed as the vaccine. The same strain of organism was grown on mutton, beef, casein and non-autoclaved 'filtered' mutton agar for 24 hours, the growth was emulsified in 25 c.c. of normal saline, heated at 55°C for 30 minutes, carbolized to the extent of 0.5 per cent and standardized according to Brown's opacity method. After having tested the sterility of the vaccines thus prepared, both aerobically and anaerobically, 100 million organisms per kilo of body-weight were injected into rabbits (a) intravenously and (b) subcutaneously as the first dose. A second dose of 200 millions was injected after 6 days' interval by the same routes.

(e) *Experimental animals*—The animals used for the immunity experiments have been large healthy young rabbits of both sexes free from any visible signs of disease. None of them had ever been employed for any experimental work before. Only those whose sera when tested were found to be free from natural agglutinins to *B. typhosus* were actually used.

#### EXPERIMENTS I TO VI

*Experiment I*—Rabbits Nos. 1, 2, 3 and 4 were given intravenous injection of *B. typhosus* 'smooth' (Rawlings) vaccine and their floccular titre determined at the end of the 6th and 12th day after the administration of the 1st dose. (See Table I)

*Experiment II*—Rabbits Nos. 5, 6, 7 and 8 were given intravenous injection of *B. typhosus* 'smooth' (Mrs. S.) vaccine and their floccular titre determined at the end of the 6th and 12th day after the administration of the 1st dose.

*Experiment III*—Rabbits Nos. 9, 10, 11 and 12 were given subcutaneous injection of *B. typhosus* 'smooth' (Rawlings) vaccine. (See Table III)

*Experiment IV*—Rabbits Nos. 13, 14, 15 and 16 were given subcutaneous injection of *B. typhosus* 'smooth' (Mrs. S.) vaccine. (See Table IV)

Media employed for preparation of typhoid vaccine for injection into—

Rabbits Nos. 1, 5, 9 and 13. Douglas agar containing hydrolysed product of beef as the basis.

Rabbits Nos. 2, 6, 10 and 14. Douglas agar containing hydrolysed product of autoclaved mutton as the basis.

Rabbits Nos. 3, 7, 11 and 15. Douglas agar containing hydrolysed product of non-autoclaved mutton as the basis.

Rabbits Nos. 4, 8, 12 and 16. Douglas agar containing hydrolysed product of casein as the basis.

*Experiment V*—Incorporates the results of agglutination tests for the 'heat stable' or small flaking, granular content of immune sera of Rabbits Nos. 6, 7, 8 and 13, 14, 15 and 16, immunized with *B. typhosus* 'smooth' (Mrs. S.) vaccine. (See Table V)

*Experiment VI*—Rabbits Nos. 17, 18, 19 and 20 were given intravenous injection of a 'rough' typhoid vaccine (Mrs. S. rough strain) and their floccular

and granular titre estimated at the end of the 12th day after the administration of the 1st dose (See Table VI)

TABLE V

*Agglutination tests with Rabbit sera employing B typhosus 'Smooth' (Mrs S) Vaccine*

Rabbit No	Nature of medium	Vaccine	Route of administration	Granular Agglutination Titre	
				2 hrs at 55°C in water-bath	4 hrs at 55°C in water-bath
R 6	Mutton	<i>B. typhosus</i> 'Smooth' 'Mrs S' vaccine	Intravenous	+++ 1 250	+ 1 1250
R 7	'Filtered' mutton	Do	Do	+++ 1 250	++ 1 500
R 8	Casein	Do	Do	+++ 1 250	+++ 1 250
R 13	Beef	Do	Subcutaneous	+++ 1 250	+ 1 500
R 14	Mutton	Do	Do	+++ 1 250	+++ 1 250
R 15	'Filtered' mutton	Do	Do	+++ 1 250	+++ 1 250
R 16	Casein	Do	Do	+++ 1 250	+ 1 500

— = No agglutination  
 +++ = Total minus agglutination of Dreyer  
 ++ = Standard " " "  
 + = Trace " " "

TABLE VI

*Agglutination tests with Rabbit sera employing B typhosus 'Rough' (Mrs S) Vaccine*

Rabbit No	Nature of medium	Vaccine	Route of administration	Type of agglutination	Agglutination titre
R 17	Beef	<i>B. typhosus</i> 'Rough' 'Mrs S' vaccine	Intravenous	Floccular	+++ 1 12500
				Granular	nil
R 18	Mutton	Do	Do	Floccular	++ 1 25000
				Granular	nil
R 19	Casein	Do	Do	Floccular	++ 1 25000
				Granular	nil
R 20	'Filtered' mutton	Do	Do	Floccular	+++ 1 5000
				Granular	nil

— = No agglutination  
 +++ = Total minus agglutination of Dreyer  
 ++ = Standard " " "  
 + = Trace " " "

## EXPERIMENT VII AGGLUTINATION TEST WITH SERA OF TYPHOID CASES

It would perhaps be of interest to give the agglutination results of sera of patients convalescent from typhoid fever, in order to give an idea of the 'heat stable' agglutinin content, following actual infection with *B typhosus*. In every one of these cases, the causal organism was isolated from blood, faeces or urine.

Heat labile agglutinins could be demonstrated in all the twelve cases examined. In some cases the titre was as high as 1 5000, in others as low as 1 250. Heat stable agglutinins, on the other hand, could only be demonstrated in 25 per cent of convalescent cases, the highest titre in the positive cases being 1 125, the lowest 1 50. It is, however, possible that in a considerable percentage of cases, the appearance and content of heat stable agglutinins in the blood of patients may be influenced by the clinical course of the disease. One may, therefore, attribute this comparatively low figure to the fact that the sera were taken from convalescent cases and not from patients actually suffering from an attack of typhoid fever.

TABLE VII

*Agglutination tests with typhoid convalescent sera*

Convalescent No	<i>B typhosus</i> isolated or not	Floccular agglutination titre 2 hrs at 55°C in water-bath	Granular Agglutination Titre			
			2 hrs incubation in water-bath at 55°C	4 hrs incubation in water-bath at 55°C	4 hrs incubation in water-bath at 55°C	Reading taken 24 hrs later
11	Isolated	+++ 1 2500	+++ 1 25	+ 1 125	+ 1 125	
12	Do	+++ 1 250	nil	nil	nil	
13	Do	++ 1 5000	nil	nil	nil	
14	Do	++ 1 2500	nil	nil	nil	
15	Do	+++ 1 2500	nil	nil	nil	
27	Do	+++ 1 250	nil	++ 1 50	++ 1 50	
28	Do	+++ 1 500	nil	nil	nil	
29	Do	+++ 1 5000	+++ 1 25	+++ 1 50	+++ 1 50	
30	Do	++ 1 250	nil	nil	nil	
31	Do	+ 1 500	nil	nil	nil	
34	Do	++ 1 5000	nil	nil	nil	
35	Do	+++ 1 250	nil	nil	nil	

— = No agglutination  
 +++ = Total minus agglutination of Dreyer  
 ++ = Standard " " "  
 + = Trace " " "

## IMMUNITY EXPERIMENT (See Table VIII)

The following experiment was carried out to determine the extent of actual protection afforded against infection with *B typhosus* by the use of typhoid vaccines

Having determined the minimum lethal dose of *B typhosus* for rabbits, a test dose of live organisms was injected intravenously into all the immunized rabbits, four weeks after the last dose of vaccine had been given. A series of non-vaccinated, normal rabbits were given the same dose, and the results compared.

Test dose 24 hours old broth culture of *B typhosus* 'smooth' (Mrs S)

Minimum lethal dose = 0.9 c.c. per kilogram of body-weight

Doses injected

1	minimum lethal dose
1.5	" " doses
2	" " "
2.5	" " "

TABLE VIII

Protection following immunization with typhoid vaccines

Rabbit No	Weight	Vaccine	Route of immunization	Test dose per kilo	REMARKS
R 6	1,270 grms	<i>B typhosus</i> 'Smooth' 'Mrs S'	Intravenous	1 M L D	Alive
R 7	1,450 "	Do	"	1.5 M L D	Alive
R 8	1,430 "	Do	"	2 M L D	Alive
R 13	1,470 "	Do	Subcutaneous	1 M L D	Alive
R 14	1,500 "	Do	"	1.5 M L D	Alive
R 15	1,600 "	Do	"	2 M L D	Alive
R 16	1,770 "	Do	"	2.5 M L D	Died
R 17	1,400 "	<i>B typhosus</i> 'Rough' 'Mrs S'	Intravenous	1 M L D	Alive
R 18	1,660 "	Do	"	1.5 M L D	Died
R 19	1,530 "	Do	"	2 M L D	Alive
R 20	1,550 "	Do	"	2.5 M L D	Alive
Control 1	1,360 "	No vaccine		1 M L D	Died
Control 2	1,470 "	Do	"	2 M L D	Died
Control 3	1,520 "	Do	"	2.5 M L D	Died

Intravenous inoculation of 24 hours broth culture of *B typhosus* 'smooth,' into immunized and non-immunized rabbits, four weeks after the last dose of immunizing vaccine

#### ANALYSIS OF RESULTS

*Heat labile, 'Floccular' content of immune sera*—Analysis of the tabulated results of Experiment I shows that so far as can be judged from the small number of animals used, intravenous series, beef vaccine gives the highest 'floccular' agglutination titre, next comes autoclaved mutton, then non-autoclaved mutton and lastly casein vaccine

Experiment II practically confirms the high agglutinogenic value of beef vaccine, though unfortunately rabbit No 5, vaccinated intravenously with beef vaccine, died a couple of days before the second specimen of its immune serum could be obtained. Mutton (non-autoclaved) comes next, while casein and autoclaved mutton show practically the same floccular titre

In the subcutaneous series—Experiment III casein vaccine gives the highest floccular titre, beef and mutton vaccines are practically identical. This sudden rise of agglutinins in rabbit No 12, vaccinated subcutaneously with casein vaccine, is probably the result of individual peculiarity of the animal, as is quite a common occurrence in experimental work. In Experiment IV, beef, mutton and casein vaccines, administered subcutaneously, cause practically the same floccular response, while non-autoclaved 'filtered' mutton shows the highest titre

*Heat stable, 'granular' content of immune sera (Experiment V* See Table V)—Subcutaneous or intravenous immunization of rabbits with killed typhoid vaccines stimulates the production of heat stable agglutinins, provided a 'smooth' strain is employed in the preparation of vaccines

As compared to the heat labile content, the titre of heat stable agglutinins in such immune sera is considerably less. It can, however, be easily demonstrated by using a sufficiently sensitive strain in the preparation of alcoholized emulsions. The route of administration of vaccine, whether subcutaneous or intravenous, makes no appreciable difference in increasing the granular titre

*'Floccular' and 'granular' titre of sera of rabbits immunized intravenously with a rough typhoid vaccine (Experiment VI* See Table VI)—Experiment VI gives the results of intravenous injection of a 'rough' typhoid vaccine prepared from 'Mrs S' rough strain, grown on beef, mutton and casein media. Whereas a very high floccular titre is obtained by means of this vaccine in all the four rabbits, none of them shows even a trace of heat stable agglutinins, in dilutions as low as 1/125 with a smooth alcoholized emulsion

*Protection following vaccination*—It is unsafe to draw any definite conclusions from a smaller number of experiments. The following are, however, published with a view that, when combined with the results of other investigators in the same field, they may prove useful in solving a more or less debatable problem

By using rabbits as experimental animals a certain amount of protection can be demonstrated in subjects immunized with typhoid vaccines. Whereas non-vaccinated rabbits die with one M L D, vaccinated rabbits survive after  $1\frac{1}{2}$  and 2 M L doses. Route of administration of vaccine, whether intravenous or subcutaneous, makes no appreciable difference in the extent of protection afforded. 1,  $1\frac{1}{2}$  and 2 M L doses protect the intravenously immunized rabbits to the same extent as the subcutaneous series.

It cannot be concluded from the above experiment that vaccines prepared from the rough strains give little or no protection. It was, however, noted that whereas rabbits immunized with a smooth strain did not look very ill after the test dose, those immunized with the rough vaccine looked decidedly more seedy and ill.

### SUMMARY

Typhoid portion of the Army T A B vaccine has been investigated regarding its antigenic and immunizing value. It has been found to be capable of producing both heat labile and heat stable agglutinins in the sera of immunized rabbits.

Increased resistance to infection (with *B. typhosus*) following immunization has been demonstrated in the case of vaccinated rabbits.

I am highly indebted to Major R. F. Bridges, R A M C, Commanding Enteric Convalescent Depot, for his very kindly supplying me with sera of known cases of typhoid fever and strain Ty 8 'o'.

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# GERMICIDAL FILTRATION AND ITS APPLICATION IN THE MANUFACTURE OF MEDIA

BY

LIEUT M L AHUJA, M D, CH B, I M S,  
*Central Research Institute, Kasauli*

[Received for publication, March 5, 1929]

IN the preparation of nutrient media, autoclave sterilization has so far been an almost indispensable factor. Products of hydrolysis of mutton, beef, etc., have to be subjected for varying periods, to temperatures of 120°C or 130°C, to clear and sterilize the media.

In doing so, however, some of the essential properties of these nutritional products must be inevitably lost. Because of this and the possibility of achieving an economic result in the manufacture of media, and consequently vaccines, an experiment was undertaken to prepare a medium, in which heat sterilization of the nutrient material was done away with, and secondly to compare the yield on this medium with that obtained from the same, but autoclaved medium.

## *Preparation of medium*

Ordinary double strength Douglas broth was prepared, employing mutton as the basis—no autoclave sterilization, however, being employed at any stage. After having adjusted its reaction to pH 7.6, it was strained through filter paper and the filtrate passed through a sterilized asbestos filter, into a vacuum flask, connected to a Geryk pump, capable of producing a pressure of at least 20 lbs. The filtrate was then mixed aseptically with equal quantities of 6 per cent agar suspension and tubed or plated as desired.

An easier and equally efficient substitute to use in place of 6 per cent agar, is desiccated agar-agar jelly which offers no difficulty in dissolving in water, and has the additional merit of not requiring to be filtered in order to give a clear medium. (For preparation, see Cunningham, *Indian Journal Medical Research*, 1918, Volume XIV.)



# EXPERIMENTS TO NOTE THE LARVICIDAL EFFECTS, IF ANY, OF SODIUM NITRATE, POTASSIUM NITRATE, AND MAGNESIUM SULPHATE IN NATURAL WATERS

BY

MILITARY ASSISTANT SURGEON E S FEEGRADE,  
*Harcourt Butler Institute of Public Health, Rangoon*

[Received for publication, March 12, 1929]

THESE experiments were carried out at the suggestion of the Director, Harcourt Butler Institute of Public Health, with a view to ascertaining whether any special salts, commonly found in water in Nature, have any deterrent or larvicidal qualities, either alone or in conjunction with each other

Water for these experiments was obtained from the tank north of the Harcourt Butler Institute of Public Health, Rangoon, and before commencing the experiments a sample was analysed at the end of April 1928, and thereafter experiments were conducted from the beginning of May 1928 to about the middle of June of the same year. The analysis, shown in parts per 100,000, was as follows —

Silica and insoluble matter	0 08
Oxide of iron and alumina	1 12
Calcium carbonate	4 36
Magnesium „	1 49
Sodium „	0 91
„ chloride	8 07
„ sulphate	3 78
„ nitrate	0 48
Organic matter	1 11

Mature larvæ of *Aedes argenteus* were used, and the water was always taken from the same spot in the tank,—the quantity used in all these experiments being 1,000 c c in glass jars

A graduated increase of potassium nitrate from 0.2 to 25 grammes, added to the water, produced no fatal results,—on the contrary, the larvæ went on to

pupation, and even when the maximum quantity of 30 grammes was reached, the lethal effect on larvæ was spread over a period of 48 hours

Thereafter, as a separate experiment, sodium nitrate as in the case of potassium nitrate,—the same measures being employed,—was added up to 30 grammes without having the least effect on the larvæ, and as the salt was obviously present in greater excess than would be the case in nature experiments to ascertain the minimum lethal quantity were discontinued

Next, a combination of both these salts was considered, and the comparatively small quantity of 10 grammes of potassium nitrate with 8 grammes of sodium nitrate per litre proved fatal in 48 hours

As these quantities and proportions were still in excess of natural conditions magnesium sulphate was introduced in conjunction with potassium nitrate, and the finding here was that with 8 grammes of the former and 10 of the latter, larvæ died in 17 hours,—so far the best results obtained in these experiments

These experiments were brought to a close as the writer was deputed to carry out a malaria survey, but it is intended to continue later on similar lines with other salts and different combinations thereof

# OBSERVATIONS ON THE MARKINGS OF *A JAMESII* THEOBALD

BY

MILITARY ASSISTANT SURGEON E S FEEGRADE,  
*Harcourt Butler Institute of Public Health, Rangoon*

[Received for publication, March 12, 1929]

SPECIMENS of the species of *A jamesii* Theobald were found amongst the mosquito fauna of Kyaukpyu, Burma, during a survey undertaken in the malarial season of 1927. These specimens, though few in number, were found not to coincide, in respect of wing markings and the extent of abdominal scaling, with the descriptions given by F V Theobald and included in the 2nd Edition of the 'Monograph of the Anopheline Mosquitoes of India' by James and Liston (1911), nor even with the more detailed account by Major G Covell, R M S, in his publication in the 'Indian Journal of Medical Research,' Vol XLV, No 4, April 1927. For this reason I identified those found at Kyaukpyu provisionally as *A jamesii*.

After a study of specimens, from Kyaukpyu, Mawlaik, and from the Central Malaria Bureau, Kasauli, collected at Chittagong, I felt assured that the differences observed by me were a constant feature of the species as against the description given by Theobald and Covell.

In the Table below are compared the respective observations of the aforementioned workers and myself.

TABLE

	Theobald	Covell	Writer
Costa	Six black-scaled areas, two being small and near the origin	Six dark areas the pale areas on the outer third being at least equal in extent to the dark ones whilst the inner quarter is chiefly pale	Seven dark areas, the three basal being small and the four distal greatly extended

TABLE.—*contd*

	Theobald	Covell	Writer
2nd Longitudinal vein	One small dark area on the posterior branch	One dark spot on the posterior branch	Two small dark areas on the posterior branch
3rd Longitudinal vein	Two dark-scaled areas at the beginning and one at its termination	Entirely pale except for a small dark area towards the base and a black spot at the apex	Pale at the beginning two black spots close thereto and one at its termination in an otherwise pale vein
Abdomen	Clothed with whitish hairs on the first seven segments and with scales and hairs on the eighth segment and genital processes	Abdomen clothed with golden hairs on the dorsum of all segments with a conspicuous dense covering of golden hairs and scales on the last two segments and genital processes	Both surfaces covered with golden hairs and dense golden scales on genitalia Dorsum of seventh and eighth segments with many golden scales and a few on the sixth, fifth and fourth decreasing in numbers as the fourth is approached Ventral surface of seventh and eighth segments with few scales

The differences noted are not variations but actual markings of *A. jamesii*. My reason for bringing these points to notice is to remove the possibility of error as to their identity in future.

# SOME MORE VARIANTS OF *A. FULIGINOSUS* GILES, FROM BURMA

BY

MILITARY ASSISTANT SURGEON E. S. FEEGRADE,  
*Harcourt Butler Institute of Public Health, Rangoon*

[Received for publication, March 12 1929]

IN his 'Note on the variations of the hind tarsal markings of *Anopheles fuliginosus* Giles, and *Anopheles ramsayi* Covell' by Major G. Covell, I.M.S., Central Malaria Bureau, Kasauli, there is a complete review of *Anopheles fuliginosus* in regard to its identity and its many variations, since its first description by Giles in 1901. The decision to identify the variant var *nagpori* with *A. fuliginosus* var *adiei* and later to declare var *nagpori* and var *adiei* as synonyms of *A. fuliginosus* Giles, by Christophers in 1916 and 1924 respectively, remove the possibility of error in considering variants as new species, for it has been found that many variations prevail in different species.

The observations on variations of *A. fuliginosus* have so far been confined to the bands on the palps and the extent of white coloration involving the hind tarsi. In re-studying specimens of *A. fuliginosus* Giles, collected in the plains and hills of Burma, for the recently established Malaria Bureau, Rangoon, it has been found that, apart from the variations in the palpal bands and hind tarsal markings as noted by several other workers, other variations in the palps as also in the scaling of wing veins prevail.

In the case of the palpal bands in otherwise normal type forms of *A. fuliginosus* Giles, from Burma, it was found that a flash of white scales occupied the space between the 2nd and 3rd white bands. Whilst in others, besides the flash of white scales on the palps, other variations existed, for instance, the 1st longitudinal vein had eight white spots instead of six and the 4th vein had no white scales at its bifurcation.

In others, again, where the third hind tarsus were picked out in black at their apical ends, the 3rd vein was entirely black-scaled except for a small white spot near its beginning.

The winter forms of *A. fuliginosus* Giles, of the plains and hills of Burma have invariably been found to have both black and white scales which stand out more clearly in winter than in summer, and the winter forms obtained from the hills are even more distinctly marked than are those from the plains

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## TWO SPECIES OF *CULICOIDES* WHICH FEED ON MAN

BY

R O A SMITH, D T M, I M D  
(From the Kala-azar Commission)

[Received for publication March 18, 1929]

THE fact that cultivators in many villages in Assam have to protect themselves with smoke from the bites of midges while working in their fields is well known. Attached to their waist bands they use ropes of twisted straw which are kept burning, the smoke envelopes each person and protects his bare body.

When surveying a village in the Burnihat area for sand-flies in connection with the investigation of kala-azar, the author and his insect collector were attacked by midges, some of which were secured.

On examination it was found that they were of two species of *Culicoides* which, as far as can be ascertained, have not been described before.

Some experiments with *Culicoides* were done in Calcutta in 1924. Altogether 9 species were secured in and around Calcutta, two of these were new, and were named *C. bimaculicosta* and *C. poeciloptera*, respectively, by the Imperial Entomologist, Pusa. None of them, however, would feed in captivity on a warm-blooded host.

On visiting the cattle shed attached to one of the houses in the village, referred to above, large numbers of *Culicoides* and one species of *Lasiohelea* were captured feeding on the cattle there. *C. ovisstoma* and *C. anopheles* were present in large numbers, as well as three other species which have not been described before, of which two resemble *C. ovisstoma* and one, the species described here, for which the name *Culicoides actoni* is suggested.

No males of the species to be described have been captured up to now, but it is hoped that some may be secured in the near future.

### *Culicoides actoni*, sp. nov.

Length of body	0.72 mm
Length of wing	0.69 mm
Breadth of wing	0.32 mm

*Head*—Black, clypeus and occiput sparsely clothed with brown hairs. Maxillæ and mandibles highly chitinated and armed with several teeth. Eyes

contiguous dorsally Dorsum of thorax slate coloured, sparsely clothed with brown hairs Humeral pits linear Abdomen dark brown above lighter beneath Halteres cream coloured Scutellum and post scutellum black

*Antennæ*—Yellow brown in colour, composed of 15 segments *Torus* dark brown and covered with minute hairs with a few long ones at the upper margin Segments 3 to 10 barrel-shaped getting progressively longer Segments 11 to 15 elongated, 15th the longest about six times as long as broad

*Palps*—Dark brown in colour, composed of 5 segments First the smallest, 2nd the longest, 3rd swollen about the upper third, and bearing a large sensory depression Fourth and 5th segments are nearly equal in size The fifth carries about four or five long hairs on its rounded extremity (Plate XIII, fig 5)

*Legs*—Yellow brown in colour Anterior tibiæ armed with spines Hind tibiæ with a row of stout spines basally First tarsal segments about three times as long as the 2nd

*Wings*—Wings are characteristic In the centre of the upper margin of the wing is a dark black area which involves the termination of the costa and the 1st and 3rd veins This area is bounded on each side by a light area roughly circular in outline The outer end of the wing above the upper branch of the 4th vein is taken up by a clear space Below the superior branch of the 4th vein the wing membrane is marked irregularly with light and dark areas (Plate XIII, fig 4) The wing is completely covered with microscopic setæ with a few large decumbent hairs situated in the outer end of the wing mostly round the termination of the superior branch of the 4th vein

*Venation*—The 1st and 3rd veins form two cells fairly equal in size The transverse vein is slightly oblique Bifurcation of the 4th vein takes place about the centre of the wing in line with the conspicuous dark area on the wing margin

*Spermathecae* two in number—globular, about 31  $\mu$  in length and 29  $\mu$  in breadth (Plate XIII, fig 6)

### ***Culicoides raripalpis*, sp. nov**

Small dark coloured fly

Length of body	1.2 mm
Length of wing	1.0 mm
Breadth of wing	0.39 mm

*Head*—Black, eyes contiguous above Mouth parts highly chitimized and adapted for piercing

*Antennæ* composed of 15 segments *Torus* dark brown, flagellum light brown The last five segments are elongated and the 15th the longest, six times as long as broad

*Palps*—Composed of five segments, 1st very short and small, 2nd elongated and slightly swollen in its upper half resembling an Indian club, 3rd as long as second and swollen in its upper half, no large sensory depression is seen on this

segment, some small pits are seen scattered over the swollen part. Fourth and 5th segments short, the 5th with 2 or 3 long hairs on its rounded extremities (Plate XIII, fig 2). Dorsum of thorax dark brown with a few rows of hairs. Scutellum and post-scutellum black.

*Wings*—The wing is dark coloured with two light areas at the costal margin. One involving the transverse vein and the other at the termination of the 3rd vein. The 1st and 3rd veins form two cells, the distal larger and longer than the proximal. The wing is completely covered with microtrichia with a few macrotrichia in the upper and outer third of the wing (Plate XIII, fig 1). Halteres brownish with cream coloured tops.

*Legs*—Dark brown with light areas below the knee joints. Bases of hind tibiae light coloured and armed with a comb of short spines. First tarsal segment 3 times the length of 2nd.

*Abdomen*—Black.

*Spermatheca*—Three in number, of unequal size and different shapes. The ducts are not chitinized (Plate XIII, fig 3).

EXPLANATION OF PLATE XIII

- Fig 1 Wing of *C raripalpis* ♀ .  
„ 2 Palp of *C. raripalpis* ♀ .  
„ 3 Spermathecæ of *C raripalpis*  
„ 4 Wing of *C actoni* ♀ .  
„ 5 Palp of *C actoni* ♀ .  
„ 6 Spermathecæ of *C actoni*

Figures drawn by camera lucida from balsam mounts

PLATE XIII



Fig 1



Fig 2

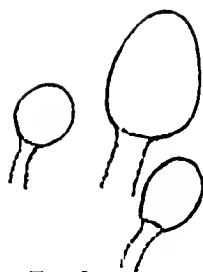


Fig 3



Fig 4



Fig 5

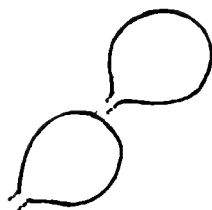


Fig 6



# A REVISION OF THE CULICINE MOSQUITOES OF INDIA

## Part XXVI.

### THE GENERA *HEIZMANNIA*, *HAEMAGOGUS*, *TOPOMYIA*, AND *MEGARHINUS*

BY

P J BARRAUD, FRS, FZS, FLS,  
*Entomologist to the Malaria Survey of India, Kasauli*

[Received for publication, March 19, 1929]

THIS paper concludes the present Revision of the Culicine mosquitoes of India, or the tribe Culicini, as regards the adult stage of these insects, as far as they are known at present. The information collected during the preparation of these papers will be embodied in a volume in the Fauna of India series, by Colonel Christophers and the writer, which is now in preparation. The larval stages of a large number of species still remain undescribed and endeavour will be made, as time permits, to prepare descriptions and figures of these for publication.

I wish to take this opportunity of again expressing my thanks to Colonel S R Christophers, CIE, OBE, FRS, IMS, for his unfailing encouragement and help during the progress of this inquiry, which at times has been interrupted for long periods owing to the pressure of more important work. My thanks are also due to Colonel J D Graham, CIL, IMS, Public Health Commissioner with the Government of India, Secretary to the Scientific Advisory Board, and to the Governing Body, of the Indian Research Fund Association under whose aegis the investigations have been carried on, to Major J A Sinton, VC, OBE, IMS, Director of the Malaria Survey of India, for his help and kindness since I joined the Survey in May 1927, and especially to Mr F W Edwards of the British Museum for his assistance in the identification of a number of specimens during the early stages of the inquiry, and for his ready help at all times, more particularly in the re-examination of type specimens in the National collection, at my request.

## Genus HEIZMANNIA Ludl

*Heizmannia*, Ludlow, 1905, *Can Ent*, Vol XXXVII, p 130

*Bolbodeomyia*, Theobald, 1910, *Rec Ind Mus*, Vol IV, p 31

This is a genus of small dark-coloured mosquitoes distinguished by the presence of a well-marked tuft of setae, or small hairs, on the postnotum\*. They are found chiefly in heavily forested localities where the larvae live in tree-holes and bamboo stumps during the monsoon. Other characters common to the Indian species may be summarised as follows —palpi short in both sexes, last two segments of antennae of the males very long, the other flagellar segments very short, in all the species where the males are known, prothoracic lobes large and in some species practically touching behind the head, mesonotum without bristles on the larger part of the dorsum, this part covered with broad recumbent scales, causing a superficial resemblance to the larger species of *Rachionotomyia*, these scales and the flat scales on the head and scutellum have, in many species, a bright metallic lustre, pleural bristles reduced in number, usually 3 or 4 proepimeral, no spiracular or upper sterno-pleural, 1 or 2 lower and a moderate number of upper mesepimeral. Male genitalia (Figs 1 to 10) characteristic of the genus and of peculiar structure, side-piece comparatively short, wide, and rounded, with a subapical lobe bearing 1 or 2 strong spines, and usually several hair tufts springing from near the base on the inner side, in some species these are remarkably long, matted and twisted. Arising also from the inner side of the side-piece towards the sternal side is a very peculiar process apparently representing the 'hairpago' or 'claspette', the apical part or 'blade' of this structure is exceedingly transparent and divided in some species into several leaflets, the form of which is very difficult to see except in heavily stained preparations. Some drawings have been prepared from suitably stained dissections and are reproduced on one of the accompanying plates. Drawings of the clasper of five species are also given from which it will be seen that the form of this is also specialised and of different shape in each species. The parameral plates and lateral plates of the phallosome are small, the latter with numerous small sharp teeth, chitinizations of the anal segment with a single blunt tooth at the crown. In two species the ninth sternite is unusually large, being about the length of the side-piece. The structure of the genitalia appears to indicate a relationship to the genus *Aedes* in the wide sense, as pointed out by Edwards. In the examination of the genitalia the reader should refer to Plates IX and X of Vol X of this *Journal*, where figures were given illustrating Edwards' Synopsis of Oriental Culicine Mosquitoes, and also to the synoptic table given below for the identification of the males of the Indian species.

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\*The only other Indian mosquito in which small hairs are present on the postnotum is *Armigeres (Leicosterna) flavus* (Leic), a large brownish-yellow species which is not likely to be confused with any species of *Heizmannia*.



*Synoptic table for the identification of the Indian species of Heizmannia*

Females

- |   |  |                        |
|---|--|------------------------|
| 1 | Proboscis more than $1\frac{1}{2}$ times the length of the fore femur  | <i>indica</i>          |
|   | Proboscis only slightly, if at all, longer than the fore femur   | 2                      |
| 2 | A continuous lateral white border to the mesonotum passing over the wing roots to the lateral lobes of the scutellum   | <i>edwardsi</i> sp. n. |
|   | Mesonotum without a continuous white border  | 3                      |
| 3 | A large patch of silvery scales on the mid lobe of the scutellum extending anteriorly on to the mesonotum, hind femur entirely pale on the basal half  | <i>himalayensis</i>    |
|   | Scutellum with only a small patch of silvery scales on the mid lobe, or with none, hind femur dark along the dorsal edge from the base to the knee joint   | 4                      |
| 4 | Prothoracic lobes entirely dark-scaled   | <i>funerea</i>         |
|   | Prothoracic lobes with some white or silvery scales  | 5                      |
| 5 | Plume scales on anterior fork veins very narrow and linear   | 6                      |
|   | Plume scales on anterior fork veins rather narrow but ligulate in shape  | 7                      |
| 6 | Mesonotal scales dull greyish brown with little or no metallic lustre, prothoracic lobes large and touching behind the head  | <i>completa</i>        |
|   | Mesonotal scales with metallic green or bluish-green lustre, prothoracic lobes smaller, more or less separated, and covered with silvery-white scales  | <i>chandi</i>          |
|   | Mesonotal scales with dark blue metallic lustre, prothoracic lobes more or less separated and with white scales along the front edge only  | <i>greeni</i>          |
| 7 | Mesonotal scales with deep blue metallic lustre, proboscis slightly longer than the fore femur, prothoracic lobes large and nearly touching behind the head, with white scales on the outer anterior parts | <i>metallica</i>       |

Females—*contd*

Mesonotal scales blackish with bronzy metallic sheen, proboscis shorter than the fore femur, prothoracic lobes more separated, with silvery-white scales anteriorly *covelli* sp n

Mesonotal scales with bright green metallic lustre, proboscis about the same length as the fore femur, prothoracic lobes rather large, somewhat closely approximated, and covered with silvery-white scales *viridis* sp n

## Males

- |   |  |                     |
|---|--|---------------------|
| 1 | Prothoracic lobes entirely dark-scaled   | <i>funerea</i>      |
|   | Prothoracic lobes with some white scales   | 2                   |
| 2 | Ninth sternite very large and much more than half the length of the side-piece   | 3.                  |
|   | Ninth sternite not more than half the length of the side-piece   | 4                   |
| 3 | Proboscis longer than the fore femur*†   | <i>indica</i>       |
|   | Proboscis shorter than the fore femur†   | <i>covelli</i> sp n |
| 4 | Clasper not markedly expanded apically and divided nearly to the base into two separate arms*  | <i>complex</i>      |
|   | Clasper expanded apically and not divided into two separate arms   | 5                   |
| 5 | Subapical lobe of side-piece with two strong spines*   | <i>greeni</i>       |
|   | Subapical lobe with only one strong spine  | 6                   |
| 6 | Clasper greatly expanded apically, side-piece with tufts of very long hairs from the base subapical lobe produced into an arm directed basally with a single strong spine at the extremity*† | <i>himalayensis</i> |
|   | Clasper less expanded, side-piece with tufts of rather short hairs all about the same length, subapical lobe not produced into a long arm  | 7                   |

\* *Vide* illustrations of male genitalia on Plates IX and X of Vol X of this *Journal*

† *Vide* illustrations on the accompanying Plate

Males—*contd*

- 7 Side-piece with a large dense tuft of rather short twisted hairs near the base, clasper with an elbow-like projection near its base\*† *chandi*

Side-piece without any large dense tufts of hairs, clasper without any elbow-like projection near its base† *metallica*

The males of *H. edwardsi* and *H. viridis* sp. n. are at present unknown

**Heizmannia complex (Theo)**

*Bolbadeomyia complex*, Theobald, 1910, *Rev. Ina Mus.*, Vol. IV, p. 31

Through the kindness of the Director, Zoological Survey of India, I have been able to examine the type male and female of this species, the genitalia of the male, however, are in the British Museum. There are two female specimens in the Malaria Survey of India collection, Kasauli, from Assam (Nongpoh, vii 1922, and iii 1927 (Barraud)) which appear to be the same species, and the following notes have been made from an examination of these four specimens. It should be mentioned that both the types, which were collected more than twenty years ago, are now in rather poor condition. General colouration dark brownish, proboscis about the length of the fore femur, mesonotal scales dull greyish brown with little or no metallic lustre, prothoracic lobes large and practically touching behind the head, with white scales anteriorly, scutellar scales dark brown like those on the mesonotum, abdomen deep brown with small lateral pale markings, rather square in shape on segments 2 and 3, small and triangular on the remaining segments and not forming bands over the dorsum, plume scales on the anterior fork veins narrow and linear.

The shape of the clasper of the male genitalia differs from that of any other known species of the genus being divided nearly to the base into two separate arms (*vide* Figs. 18 to 20 on Plate IX of Vol. X of this *Journal*).

The types are in the Indian Museum, both from Dawna Hills, Lower Burma, iii 1908 (*Innaudale*).

**Heizmannia funerea (Leic)**

*Wyeomyia funerea*, Leicester, 1908, *Cul. Malaya*, p. 252

This differs from all the other Indian species in having the large prothoracic lobes entirely covered with dark scales. I have not seen any specimens, but one female of probably this species is mentioned in some mss. notes kindly supplied

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\* *Id.* illustrations of male genitalia on Plates IX and X of Vol. X of this *Journal*  
 † *Id.* illustrations on the accompanying Plate.

to me by Mr F W Edwards, caught biting in the day time at Meenglas, Jalpaiguri, North Bengal (*M O T Iyengar*)

The male genitalia do not appear to have been figured but amongst a large number of micro-photographs taken by Surgeon Commander O'Flynn, R N, kindly sent to me by Surgeon Commander D H C Given, R N, of H M Naval Base, Singapore, is one of a slide preparation bearing this name. From this it appears that the structure of the hypopygium is similar to that of *H indica* and *H covelli* sp n, but there are differences in the form of the hair tufts and in the shape of the clasper.

*W funerea* was described from a single female taken in jungle 6 miles from Kuala Lumpur, Malay Peninsula (*Leicester*)

### ***Heizmannia edwardsi* sp n**

This species differs from any I have seen in the presence of a continuous white scaled margin to the mesonotum continued over the wing roots and terminating on the lateral lobe of the scutellum on each side.

Description of female —*Head* a wide area of flat grey scales on the vertex, a narrow pale border to the eyes and a large pale patch at each side of the head, a few dark upright scales on the nape, tori with some pale scales on the inner sides, flagellar segments and hairs dark, clypeus pale grey, palpi and proboscis black with a dull dark bluish sheen, palpi about one-quarter the length of the proboscis. *Thorax* no bristles on the larger part of the dorsum of the mesonotum, this part covered with broad recumbent brownish-grey scales of a very dark shade, without any metallic lustre, a border of white scales along the sides of the mesonotum continued over the wing roots to the lateral lobes of the scutellum, the white scales over the wing roots very large, prothoracic lobes rather large but separated, covered with dull white scales, scutellar scales broad and flat, white on the lateral lobes but apparently all dark on the mid lobe, a well-marked tuft of setae on the postnotum, pleurae almost entirely covered with white flat scales (not silvery) including pro-epimera, post-spiracular area, sterno-pleura, and larger part of mesepimeron. *Wings* dark-scaled, plume scales on anterior fork veins narrow but ligulate in shape, wing length 3.8 to 4 mm. *Legs* tibiae and tarsi brownish-black without any pale markings, fore and mid femora dark on the anterior surface, pale posteriorly for nearly the whole length, hind femur white along both sides except along the dorsal edge but the knee joint dark. *Abdomen* deep brownish-black rather than blue-black, rather large and irregular lateral white patches, widest towards the apex of each segment, not forming bands over the dorsum.

Two co-type females are in the Malaria Survey of India collection, Kasauli, from Yellapur, North Kanara, October 1921, one caught in forest on the 6th, and one bred from larva found in hollow bamboo (*Barraud*). The male is at present unknown.

**Heizmannia indica** (Theo)

*Phantomia indica*, Theobald, 1905, *Ann Mus Nat Hung*, Vol III, p 115,  
and *Mou Cul*, Vol IV, p 601, 1907

This species is fairly easily distinguished by the unusual length of the proboscis which is from one and a quarter to one and a half times the length of the fore femur. The mesonotal scales are distinctly greenish-blue with metallic lustre, those on the abdomen bluish-black, the lateral basal white triangular markings on the latter do not usually extend on to the dorsum to form narrow bands as is the case in several other species. The scutellar scales are dark greenish like those on the mesonotum, a few scales on the mid lobe may appear light when viewed in certain positions but there is no large silvery area as in *H himalayensis*. The prothoracic lobes are more or less separated with silvery scales on the upper and anterior parts, dark scales below. The plume scales on the anterior fork veins are rather narrow but ligulate in shape. Hind femur dark along the dorsal edge from the base to the knee.

The male genitalia are remarkable owing to the enormous development of the ninth sternite which is hood-like and as long as the side-piece. Figures of the genitalia were given in Edwards' synopsis Vol X, of this *Journal*, Plate X, figs 21 and 22. Figures (Nos 2 and 6) on the accompanying Plate show the shape of the clasper and of the 'harpago'.

Specimens have been examined from the following places —

North Bengal — Sukna, ix and x 1922, bred from larvae found in bamboos and tree-holes (*Barraud*), Marimbari Tea Estate, viii 1928, caught in jungle (*Sobha Ram* collr)

Eastern Himalayas — Darjeeling, vii 1918, bred from larvae found in tree-holes (*Kimp*), Tindharia, Sureil, and Kurseong, ix and x 1922, bred from larvae found in bamboos and tree-holes (*Barraud*), Kurseong viii 1928, larvae from bamboos (*Sobha Ram* collr)

*P. indica* was described from one male from Singapore, 1902 (*Biro*). The type is in the National Museum of Hungary, Buda Pest.

**Heizmannia covelli** sp. n.

This species differs from *H. chandi*, *H. greeni* and *H. metallica*, to which it appears to be related, in the structure of the male genitalia, and in the combination of the following characteristics present in both sexes — proboscis distinctly shorter than the fore femur, mesonotal scales blackish with a bronzy metallic sheen, scutellar scales greenish black with a few silvery scales at the apex of the mid lobe, prothoracic lobes approximated but not touching behind the head, with silvery scales anteriorly, plume scales on anterior fork veins narrow but ligulate in shape. The abdomen is marked with the usual lateral triangular silvery basal patches, and there are narrow white basal bands over the dorsum on segments 6 and 7 in the female. There are 3 or 4 proepimeral bristles overlapping the spiracle, 1 or 2 lower mesepimeral and 3 or 4 bristles on the lower part of the

**sterno-pleura** The last two flagellar segments of the antenna of the male are very long as in other species, the terminal segment is about twice the length of the penultimate and the last two segments together are a little more than twice the length of the first eleven, the flagellar segments 2 to 11 are each only about twice as long as broad. Both claws of the fore and mid legs of the male have a slender sharp tooth arising from the base.

The male genitalia are remarkable for the development of hair tufts on the inner side of the side-piece (Fig 10), one of these is twisted upon itself several times at the base and is composed of a number of long stout hairs hooked at the tips. The 'harpago' is also remarkably distinct from that of any other species (Fig 7).

Two co-type males are in the Malaria Survey of India collection, Kasauli, from the Andaman Islands, vii 1926, bred from larvae found in bamboo stumps (*Major G. Covell, I M S*), also one other male from Sukna, North Bengal, ix 1922, bred from larva found in tree-hole (*Barraud*), and one allotype female from the same place, x 1922 (*Barraud*).

### ***Heizmannia viridis* sp. n.**

This most nearly resembles *H. covelli* sp. n. and some provisionally named specimens of *H. metallica* (Leic.) but differs from these and from the other Indian species as indicated in the synoptic table.

**Description of female**—*Head* a median patch of flat silvery scales on the vertex in front continued downwards between the eyes, remainder of the dorsal surface covered with flat blue-black scales, a patch of silvery scales low down at each side, tori, flagellar segments and hairs almost black, clypeus shining brownish-black, palpi and proboscis of the same shade, the latter equal in length to the fore femur. *Thorax* mesonotal scales with a bright green metallic lustre contrasting with the blue-black scales on the head, scutellar scales flat with a green or bluish-green metallic lustre, a few white scales in the middle of the centre lobe, prothoracic lobes rather large and fairly closely approximated and for the larger part covered with silvery scales, some black scales on the lower and posterior parts, a well-marked tuft of setae on the postnotum. Integument of pleurae black with patches of flat silvery scales on the sterno-pleura, mesepimeron, prosternum and coxae, 3 or 4 proepimeral bristles, one strong lower mesepimeral bristle and 4 or 5 on the lower posterior part of the sterno-pleura. *Wings* plume scales on the anterior fork veins rather narrow but ligulate in shape, wing length about 3 mm. *Legs* deep bronzy-brown, fore and mid femora paler posteriorly, hind pair more extensively pale on both surfaces, dark along the dorsal edge and around the knee-joint. *Abdomen* blue-black with the usual basal triangular white markings and more or less complete narrow basal bands over the dorsum on the terminal segments.

Three co-type females are in the Malaria Survey of India collection, Kasauli, from Yellapur, North Kanara, caught in forest, 6 x 1921 (*Barraud*), also other

females of apparently the same species from Nagargali, Bombay Deccan, viii 1921 (*Barrand*), and Malabar Coast, x 1915 (*Khazan Chand*)

### ***Heizmannia himalayensis* Edw**

*Heizmannia himalayensis*, Edwards, 1922, *Ind Jour Med Res*, Vol X, p 290

This is a fairly distinct species and appears to be a common tree-hole breeding mosquito in both the Eastern and Western Himalayas from about 500 feet up to 7,000 to 8,000 ft

Distinguishing characteristics —hind femur entirely pale on the basal half even along the dorsal edge, mesonotal scales black with bluish-black metallic lustre, a large silvery patch covering the mid lobe of the scutellum and extending anteriorly on to the posterior part of the mesonotum in the middle, proboscis about the length of the fore femur, plume scales on the anterior fork veins rather narrow but ligulate in shape, prothoracic lobes slightly separated with silvery scales covering the larger part, basal triangular lateral silvery patches on the abdomen, and narrow basal white bands over the dorsum on four or five segments in the female. In the male there is a long slender tooth on the larger claw of the fore tarsi, the clasper and harpago are very distinct from those of other species (*vide* Figs 1 and 9)

Specimens have been examined from the following places —

Western Himalayas —Koti, near Kasauli, vii 1923, bred from larvae found in tree-holes (*Barrand*)

Eastern Himalayas and North Bengal —Sukna, ix 1923, larvae from tree-holes (*Barrand*), and viii 1928 (*Sobha Ram collr*), Kurseong, viii 1928, larvae from bamboos (*Sobha Ram collr*)

The species was described from a single male from Darjeeling, bred from a larva obtained from a tree-hole, vii 1918 (*Kemp*). The type is in the Indian Museum

### ***Heizmannia metallica* (Leic)**

*Wyeomyia metallica*, Leicester, 1908, *Cul Malaya* p 251

The Malaria Survey of India collection contains one male which was provisionally named as this species by Edwards in 1921, this specimen, together with one female now in the British Museum, was bred from larvae found in tree-holes at Puduradi, Malabar Coast, x 1915 (*Khazan Chand*), two more females from the same place are in the Kasauli collection

The mesonotal scales in these specimens have a deep bluish metallic lustre (not green), proboscis slightly longer than the fore femur, prothoracic lobes large and nearly meeting behind the head with silvery scales on the outer anterior parts, plume scales on the anterior fork veins ligulate in shape, abdomen with the usual large basal triangular silvery patches

In the male the first eleven flagellar segments of the antenna are very short, scarcely longer than broad, each with a very few long hairs, the last two segments with long and dense pubescence, and very long basal hair whorls

Parts of the male genitalia are illustrated on the accompanying Plate (figs 4 and 8), other figures were given in Vol X, of this *Journal*, Plate X, figs 25 to 27. The side-piece is without any tufts of very long twisted hairs, those present being quite small and composed of short hairs, the 'harpago' (fig 8) has the usual very thin transparent expansion but this is of different shape to that of any other species

*W. metallica* was described from a single female type specimen, from Bukit Kutu, Malay Peninsula (*Leicester*), which is in the British Museum

### ***Heizmannia chandi* Edw**

*Heizmannia chandi*, Edwards, 1922, *Ind Jour Med Res*, Vol X, p 291

The plume scales on the anterior fork veins are very narrow and linear in this species, as is also the case in *H. greeni* and *H. complexi*. I have not seen any authentically named specimens of *H. greeni* but there are two females in the Malaria Survey of India collection which appear to be that species and have been provisionally placed under that name. These differ from *H. chandi* in having silvery white scales on the front part of the prothoracic lobes only, whereas in the last mentioned species these lobes are almost entirely covered with scales of that kind. There is also a difference in the colouration of the mesonotal scales, in *H. chandi* these have a distinct metallic green or bluish-green lustre whereas in the provisionally named specimens of *H. greeni* they have a dark blue sheen. In *H. complexi* the mesonotal scales are dark greyish brown with little or no metallic lustre, and the prothoracic lobes are large, practically touching behind the head, which is not the case in the other two species referred to above.

The male genitalia of the three species are quite distinct as will be seen by comparing figs 3 and 5 on the accompanying Plates with figs 18 to 20 on Plate IX, and fig 30 on Plate X of Vol X of this *Journal*.

The type male, No 1282, and other males and females, are in the Malaria Survey of India collection, Kasauli, from Pudukadi, Malabar Coast, x 1915 (*Khazan Chand*), one allotype female, taken at the same time and place, is in the British Museum.

### ***Heizmannia greeni* (Theo)**

*Wyeomyia greeni*, Theobald, 1905, *Jour Bomb Nat Hist Soc*, Vol XVI, p 247

This species resembles *H. metallica* (*Leic*) and *H. chandi* Edw but differs from them as indicated in the synoptic tables. I have not seen any authentically named specimens, but there are two females in the Malaria Survey of India collection, Kasauli, from the Malabar Coast, x 1915 (*Khazan Chand*) which I have provisionally placed under this name as they agree fairly closely with the



description and are evidently distinct from the other species included in this paper. These specimens possess the following characters — proboscis about the length of the fore femur, mesonotal scales with a dark blue metallic lustre (not green as in *H. chandi*), prothoracic lobes with white scales only along the anterior edge and laterally, plume scales on the anterior fork veins very narrow and linear (not ligulate as in *H. metallica*), basal white triangular lateral patches on the abdomen not forming bands over the dorsum.

In referring to this species Edwards states that the basal flagellar segments of the antenna of the male are distinctly longer than broad, and that the sub-apical lobe of the side-piece bears two strong spines (this is not the case in any other Indian species of which the male is known).

The type male and female are in the British Museum from Peradeniya, Ceylon, 1 and 11 1902 (*Green*).

#### Genus HAEMAGOGUS Will

*Haemagogus*, Williston, 1896, *Trans Ent Soc Lond*, p 271

Two Indian mosquitoes have been provisionally referred to this genus, originally described from tropical America, but as no males of the Indian species have yet been obtained their true generic position is not definitely settled. In structure and general appearance they closely resemble the larger species of *Heizmannia*, but may be readily distinguished from these by the absence of hairs or setae on the postnotum. The prothoracic lobes are large, collar-like and closely approximated behind the head, there are no bristles on the larger part of the dorsum of the thorax, general colouration black with brilliant silvery markings, proboscis long and slender, distinctly longer than the fore femur, palpi short and only about one-sixth the length of the proboscis.

Both species appear to be rare and are confined to heavily forested localities.

#### *Haemagogus tripunctatus* (Theo)

*Stegomyia tripunctata*, Theobald, 1908, *Rec Ind Mus*, Vol II, p 288

The female of this species may be distinguished from that of *H. discrepans* referred to below by the following characters — tarsal claws of the fore and mid legs simple, wing scales rather broad, hind femur pale on the outer side nearly to the knee.

The type female is in the Indian Museum, from Lushai Hills, Assam, caught in jungle, 6 vi 1904 (*E C Macleod*). There do not appear to be any other records of the occurrence of this species in India.

#### *Haemagogus discrepans* Edw

*Haemagogus discrepans*, Edwards, 1922, *Ind Jour Med Res*, Vol X, p 291

The female may be distinguished from that of *H. tripunctatus* by the narrower wing scales, toothed claws on the fore and mid legs, and by the marking

of the hind femur, which is silvery on the outer side on about the basal half only

The type female is in the British Museum, from Pudupadi, Malabar Coast, caught in jungle, x 1915 (*Khazan Chand*), paratype females are in the Malaria Survey of India collection, Kasauli, taken at the same time and place

### Genus *TOPOMYIA* Leic

*Topomyia*, Leicester, 1908, *Cul Malaya*, p 238

*Pseudograhamia*, Theobald, 1910, *Rec Ind Mus*, Vol IV, p 26

This genus, originally described from the Malay Peninsula, is apparently very poorly represented in India, only two female specimens of one species having been found up to the present. It is probable that a further number of species exist in the forest regions of south-west India and Burma.

In the venation of the wing the Indian species resembles *Uranotaenia*, *Harpagomyia*, and *Hodgesia*, the base of vein 2, the fork of vein 5, and the tip of vein 6 being very nearly in a line at right angles to the costa, but it may be distinguished from species included in the three genera referred to by the microtrichia being of normal size, the labella not greatly enlarged, and by the wing scales being of normal shape and not emarginate at the tips. The palpi are quite short in both sexes and the antennae of the male resemble those of the female (in the Malay species), the head is flat scaled with a silvery patch on the vertex, scutellum unusually small, proboscis moderately long, slightly swollen at the tip, and apparently bent under the body when the insect is at rest, clypeus rather long and narrow, mesonotum marked with a central line of broad flat silvery scales. The pleural bristles are reduced, there being no upper sterno-pleural or lower mesepimeral, usually only two proepimeral, but on the other hand two or more spiracular bristles are present.

### *Topomyia argenteoventralis* Leic

*Topomyia argenteoventralis*, Leicester, 1908, *Cul Malaya*, p 240

*Pseudograhamia aureoventer*, Theobald, 1910, *Rec Ind Mus*, Vol IV, p 27

Theobald's type female of *P. aureoventer*, in the Indian Museum, is now in poor condition but so far as can be seen it agrees closely with Leicester's description of *T. argenteoventralis*, except that the middle of the second abdominal tergite is dark with a large silvery spot on each side, and not with 'a large patch of white scales on the dorsum'. This specimen came from Pallode, 20 miles north-east of Trivandrum, Travancore 16 xi 1908 (*Annandale*). I have seen one other female of evidently the same species from Virajpet, Coorg, 20 to 24 x 1915 (*Bainbridge Fletcher*) but this is also in poor condition.

Genus *Megastylus* R-D

*Megastylus* Richard-Denis 1827 *Mem. Soc. d'Hist. Nat.*, Vol. III, p. 413

*Tent. Hist. Nat. France* 1801 *Mem. Cit.* Vol. I, p. 244

*Mem. Soc. Denis* 1806 *Part. I. et S.* Vol. I, p. 779

*Tent. Hist. France* 1818 *Cit. Mem.* p. 49

This includes some of the largest *Megastylus* and there are a number of characters which distinguish it as a well recognised genus. The most obvious of these are the meso- and meta-tergites broad and longer than long, pronotum curved dorsally at about the middle, the anal part of the apical part more slender and tapering to a point, scutellum with the posterior margin evenly rounded, not retreating, the fore wing with a rounded to chelate apical process, the wing membrane between the veins  $r_1$  and  $r_{4+5}$  even.

Many of the species are brightly ornamented with green, blue, yellow, purple, and white scales, but there is a bright metallic lustre and in some there are blackish spots or dorsal lines on the terminal segments of the abdomen.

The male genitalia are of a distinctive structure (Figs 11 to 24 on the opposite page). The dorsal border of the ninth tergite bears rows of hairs on either side and the parts bearing these hairs may or may not form a pair of well-defined summits, lobes which sometimes broadly crescentic in shape and are flattened. The apex of the side-piece carries a long slender clasper curved with a terminal or sub-terminal appendage and surface of side-piece with a basal lobe bearing one or more long stout bristles, the lobe on one side connected to that of the other by the transverse or lateral plates of the phallosome long and narrow with a few short setae, serrations or small teeth towards the apex, parameral plates and anal apophyses moderate; or elongated, channellations of the anal segment flattened towards the crown and terminating in a tooth with a few minute hairs immediately below. On the tergal side the base of the anal segment is strongly attached to the ninth tergite.

The larvae are predaceous and when fully grown of large size, measuring 15 mm. or more in length. They are found chiefly in tree-holes and bamboo stumps during the monsoon, that of *M. splendens* has been occasionally taken from coniferous resin and water-burns.

*Summary of the characters of the adults of Indian species of Megastylus.*

1. Tarsi entirely dark in both sexes a comparatively very small and slender species wing length less than 5 mm.

*viridis*.

(Southern India and Ceylon.)

- Some of the tarsal segments with white markings larger species, wing length usually 6 mm. or more .. .. . 2.

- 2 All the tarsal segments of all the legs with white markings *albipes*  
(Western Himalayas)
- Some of the tarsal segments without white markings 3
- 3 Mesonotum with a conspicuous border of creamy scales continued from the front, along the sides, over the wing roots to the scutellum *edwardsi*  
(Western and Eastern Himalayas)
- Mesonotum without a conspicuous pale border 4
- 4 Mid and posterior cross-veins (r-m and m-cu) much nearer the base of the wing than the supernumerary cross-vein (s) (*vide* Text-figure A below), abdomen with conspicuous tufts of hairs on segments 6, 7 and 8, those on 7 black, on 8 orange 5
- Mid, posterior, and supernumerary cross-veins approximated (*vide* Text-figure B below) abdomen without conspicuous tufts of hairs 6
- 5 First abdominal tergite entirely covered with green scales, fore tibia usually pale at the apex *quasiferon*  
(Sikkim)
- First abdominal tergite blue or green in the middle, pale yellow at the sides, fore tibia dark at apex *splendens*  
(Widely distributed)
- 6 Dorsum of abdomen mainly coppery purple with narrow blue basal bands, segments 2 and 3 of mid tarsi of female mainly purple, 5th segment of hind tarsi white, 1st segment of hind tarsi of male with dense bristles beneath .. *kempfi*  
(North Kanara)
- Dorsum of abdomen mainly deep blue or deep purple with lateral pale yellow markings, segments 2 and 3 of mid tarsi of female white, 5th segment of hind tarsi dark, 1st segment of hind tarsi of male without dense bristles beneath .. *gravelyi*  
(Eastern Himalayas and Assam)

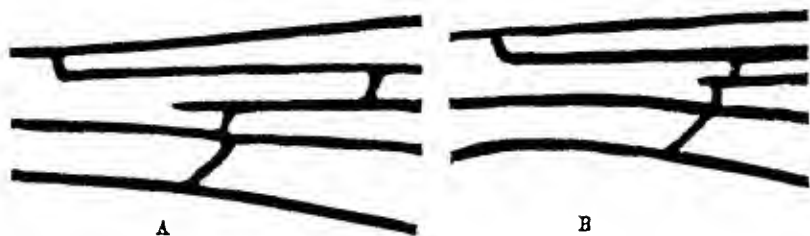


Diagram showing the position of cross-veins in the wings of *Megarhinus* spp. A *M. splendens*, B *M. kempi*. The longitudinal veins shown are, from above downwards —the 1st, stem of 2nd, base of 3rd, stem of 4th, and anterior branch of 5th

### *Megarhinus albipes* Edw

*Megarhinus albipes*, Edwards, 1922, *Ind Jour Med Res*, Vol X, p 287

Distinguishing characteristics, male and female —

A rather large species, wing length about 8 mm. Mesonotal scales brilliant metallic green, abdomen without conspicuous lateral tufts, dorsal surface dark blue to purple, with, in most specimens, narrow white basal bands on segments 2 to 5, not always complete, segments 5 to 7 with pale yellow lateral patches usually visible dorsally, 1st tergite purple in the middle, pale yellow at the sides, sternites pale golden with a narrow longitudinal median purple stripe. A pale ring at the base of the 1st tarsal segment on all the legs, most distinct on the mid and hind pair, the apical half of the 2nd segment and the whole of the 3rd, 4th and 5th on the fore legs white, except part of the 5th in some specimens, on the mid legs the 2nd to the 5th segments are entirely white, hind tarsi with white scaling at the apices of the 2nd and 3rd segments, the 4th entirely white and the base of the 5th. The wing membrane is distinctly clouded in the region of the cross-veins, the supernumerary, mid, and posterior fairly closely approximated, but rather more separated than in *M. kempi* (Text-figure B above). Ninth tergite of male genitalia narrow, apical border rounded and not produced into lobes, lateral plate of phallosome with a few small teeth, very similar to that of *M. kempi* (Fig 20), clasper similar to that of *M. splendens* (Fig 11).

The type male and female are in the British Museum, from Simla, bred from larvae found in tree-holes, 7,000 to 8,000 feet, viii 1915 (*Christophers*), paratypes bred at the same time are in the Malaria Survey of India collection, Kasauli, also other specimens from the same place, ix 1923 (*Barraud*), viii 1927 (*Puri*), and from the Krol Mountain, near Solon, Western Himalayas, bred from larvae found in tree-holes, 7,000 feet, vii viii, and ix 1923 (*Barraud*), and ix 1924 (*Barraud*).

**Megarhinus kemp** Edw

*Megarhinus (Toxorhynchites) kemp*, Edwards, 1921, *Bull Ent Res*, Vol XII, p 72

A slender species of moderate size, wing length about 6 mm or slightly less, abdomen without conspicuous lateral tufts

As the original description was made from a single male and female, neither of which was in good condition, a fairly full description is given below, based on the examination of thirty-four specimens in Malaria Survey of India collection

The straight basal part of the proboscis very stout and covered with deep purple scales with a metallic sheen, the curved apical part bluish-green or bronzy, palpi of female purple, the apical segment of the male palpi of the same colour, the basal segments mainly yellowish with purple and blue markings. Mesonotal scales brilliant green, those on the scutellum and over the wing roots bright bluish-green, scales on prothoracic lobes purple (sometimes with a bluish sheen) with a few white ones intermixed. Wings the arrangement of cross-veins is shown in Text-figure B above. Leg markings of the female femora purple along the dorsal edge, pale golden on both surfaces below, some blue and white scales forming small knee-spots, tibiae purple, some light bluish scales intermixed on the mid pair, tarsi purple with white markings showing some variation in size, a subbasal white ring on the 1st segment on all the legs, usually some white scaling at the base of the 4th segment on the fore leg, on the mid and hind legs this segment is white, 5th segment on the mid and hind legs white except for some dark scales at the tip on the mid leg, and a few dark scales at the joint between the 4th and 5th on the hind pair, on the mid leg there are sometimes a few pale scales at the base of the 2nd segment, and occasionally at the apex of the 3rd. In the male the legs are purple except for a pale ring near the base of the 1st segment on all the legs, that on the fore legs not always complete. There are numerous close-set bristles on the under side of the 1st segment of the hind tarsi for the greater part of its length. The dorsum of the abdomen is mainly coppery-purple with narrow basal bright blue bands on segments 2 to 4, or 2 to 6, white markings on the sides of the tergites produced on to the dorsum on segments 4 to 6, 1st tergite bluish-green in the middle, pale yellow at the sides, sternites pale golden but the 4th purple in the middle. In some male specimens the dorsum of the abdomen is almost entirely purple. Illustrations of parts of the male genitalia are given on the accompanying Plates, figs 18 to 21.

The type male and female came from Talewadi, near Castle Rock, North Kanara District, 3 to 10, x 1916 (*Kemp*). One of the specimens is, I believe, in the Indian Museum. The Malaria Survey of India collection, Kasauli, contains specimens from the following places in the North Kanara District—Nagargali, viii 1921 (*Barraud*), Kadra, ix 1921 (*Barraud*), and Yellapur, x 1921 (*Barraud*). All the specimens were bred from larvae or pupae found in bamboo stumps. Some

larvae brought to Kasauli, from Yellapur, a distance of about 1,600 miles, in October 1921, lived through the winter until the following April before pupating

***Megarhinus gravelyi* Edw**

*Megarhinus (Toxorhynchites) gravelyi*, Edwards, 1921, *Bull Ent Res*, Vol XII, p 73

A fairly large species, wing length 6.5 to 7 mm, abdomen without conspicuous lateral tufts of hairs

As the original description was made from a single male, not in perfect condition, a fairly full description of both sexes is given below, based on the examination of fourteen specimens in the Kasauli collection

The straight basal part of the proboscis moderately stout and covered with purple scales, the curved more slender apical part with a greenish lustre, palpi of the female purple, those of the male of the same colour but with a golden ring at the tip of the 1st segment and golden scales beneath and at the sides of parts of the 2nd and 3rd segments. Mesonotal scales bright metallic green, those on the scutellum and upper parts of the prothoracic lobes and proepimera coppery. Wing membrane with a slight darkening in the region of the cross-veins, the latter closely approximated, but rather more separated than in *M. kempti* (Text-figure B). Legs purple scaled, femora golden on both sides ventrally and towards the base, fore tibiae of the female with a large pale area on the posterior surface in the middle, mid pair with a wide nearly complete pale ring in the same position, hind pair, and all the tibiae of the male, dark, fore tarsi of the female with a wide pale ring at the base of the 1st segment, the larger part of the 2nd white, but some dark scaling near the base, variable in extent, the 3rd and 4th segments and the base of the 5th white, on the mid tarsi there is white scaling on the basal half of the 1st segment and over the whole of the 2nd, 3rd, 4th, and basal part of the 5th, but in some specimens the 5th is mainly dark on the fore and mid legs. On the hind tarsi there is a subbasal white ring on the 1st segment, and the 4th is entirely white (in one female from Haflong, Assam, this segment is mainly dark). In the male the tarsi are purple except for pale markings at the base of the 1st segment on all the legs, that on the mid leg usually forming a complete ring. The dorsum of the abdomen in some specimens is deep blue, in others deep purple, 1st tergite yellowish at the sides, small lateral pale yellow patches on segments 4 to 7, narrow basal pale yellow bands on segments 2 and 3, sometimes on segments 2 to 6, 4th sternite deep purple, the remainder pale golden with a narrow median dark longitudinal line. There seems to be some variation in the width of the clasper and in the form of the ninth tergite of the male genitalia, drawings of the claspers of two individuals, mounted in the same way, are shown in Figs 22 and 23. The ninth tergite (Fig 24) bears a pair of hairy submedian lobes, more pronounced and pointed in some specimens than in others. The lateral plate of the phallosome is without any obvious teeth.

The type male, from Pashok, Darjeeling district, 2,000 feet, 25.v to 14.vi.1916 (*F. H. Gravelly*), is in the Indian Museum. The Malaria Survey of India

## *A Revision of the Culicine Mosquitoes of India*

Collection, Kasauli, contains specimens of both sexes from —Assam, Nongpoh, vii 1922 (*Barraud*), Haflong, viii 1922 (*Barraud*), Eastern Himalayas, Tindharia and Mungpoo, x 1922 (*Barraud*) These specimens were bred from larvae and pupae found in tree-holes and bamboo stumps

### ***Megarhinus minimus* Theo**

*megarhinus minimus*, Theobald, 1905, *Jour Bomb Nat His Soc*, Vol XVI, p 237 (description of male)

*tes minimus* (Theo), Senior White, 1919, *Spol Zeyl*, Vol XI, 89 (description of female)

This is distinguished from all the other Indian species by the absence of any dark markings on the tarsi in either sex. It is comparatively very small and the wing is usually less than 5 mm long. There are no conspicuous lateral tufts on the abdomen.

The mesonotal scales have a yellowish-green metallic lustre and there are some brilliant blue and green scales over the wing roots, scutellar scales green, scales of similar colour on the upper part of the proepimera and prothoracic lobes, the latter with a bluish or purplish lustre when viewed in certain positions, scales on the lower part of the proepimera and pleurae silvery. Legs mainly yellow, femora yellowish at the base and beneath. Dorsum of the first few segments of the abdomen dark greenish purple, the remainder bright purple, indications of pale greenish bands on segments 4 to 6 or 7, some quite small yellowish lateral tufts at the apex of the 6th segment, and similar tufts of mainly black hairs on the 7th and 8th. In one female specimen examined, tergites 4 to 7 are brassy-brown.

The type male and female are in the British Museum, the former from Yatiyantota, Ceylon (*Green*), the latter, described by Senior White, from Suduganga, Matale district, Ceylon, 20 iv 1919.

The Malaria Survey of India collection contains one male from Colombo, Ceylon (*James*), one female from Yellapur, North Kanara, Bombay Presidency, x 1921, bred from larva found in bamboo stump (*Barraud*), and one female from Top Slip, 3,500 feet, Anamali Hills, Coimbatore district, Madras, 4 to 8 viii 1928 (*Shaffi*).

### ***Megarhinus edwardsi* Barr**

*Megarhinus edwardsi*, Barraud, 1924, *Ind Jour Med Res*, Vol XI, p 999

A rather large species with conspicuous lateral tufts of hairs on the terminal segments of the abdomen, wing length about 9 mm. Distinguished from all the other Indian species by the presence of a conspicuous creamy border to the mesonotum.

The straight basal part of the proboscis dark bluish, the more slender apical part bronzy, palpi dark bluish, those



yellow ring at the base of the 1st segment and a similar but wider subapical ring to the 2nd segment. A broad creamy border to the mesonotum from the front, continued along the sides, over the wing roots, to the scutellum, the disc covered with dark green metallic scales, scutellar scales dark blue in the male, greenish-blue in the female, prothoracic lobes and lower part of proepimera covered with broad creamy scales with a bluish tinge when seen in certain positions. Wings supernumerary cross-vein nearer the tip of the wing than the mid by about three times its length. Abdomen tergites dark metallic blue, the 1st, 3rd, 5th, and 6th with lateral pale yellow patches, 3rd and 5th with incomplete median pale bands, 6th with lateral tufts of pale yellow hairs, 7th with similar tufts of mainly golden hairs in the female, black in the male, 8th with small tufts of orange hairs, 2nd, 3rd, 5th, and 6th sternites pale yellow with a median longitudinal dark stripe, 4th and 7th nearly all dark. Legs tarsal markings of the male 1st segment of fore tarsi dark blue with an apical white ring, 2nd all white, 3rd with basal white ring, the 4th and 5th dark blue, basal half of 1st segment of mid tarsi yellow and the tip white, 3rd, 4th, and larger part of 5th white, hind tarsi dark except for a yellow streak on the underside of the 1st segment near the base, and a basal white ring to the 2nd. Tarsal markings of the female 1st segment of fore tarsi dark blue-green above at base, yellow beneath, the remainder and the whole of the 2nd segment white, 3rd with a few pale scales at the base, otherwise dark bluish, as are also the 4th and 5th segments, mid tarsi as in the male, hind tarsi dark bluish-green except for a subbasal yellow ring to the 1st segment, and a wide basal white ring to the 2nd.

The type male and female are at present in the British Museum, from the Krol Mountain, near Solon, Western Himalayas, bred from larvae found in tree-holes, 7,000 feet, viii 1923 (*Barraud*), one paratype female from the same place and one female from Sureil, Eastern Himalayas, x 1922 (*Barraud*), are in the Malaria Survey of India collection, Kasauli, both bred from larvae found in tree-holes.

### ***Megarhinus splendens* (Wied)**

*Culex splendens*, Wiedemann, 1819, *Zoologisches Magazin*, Band I, Stuck III, p 2

*Culex subulifer*, Doleschall, 1857, *Nat Tijds v Ned Ind*, Vol XIV, p 382

*Culex regius*, Thwaites in Tennant, 1861, *Nat Hist of Ceylon*, p 434

*Megarhinus immiscueus*, Theobald, 1901, *Mon Cul*, Vol I, p 225, nec Walker, 1860, *Jour Proc Linn Soc Lond*, Vol IV, p 91

*Megarhinus gilesi*, Theobald, 1901, 1 c p 227

*Woreestera grata*, Banks, 1906, *Phil Jour Sci*, Vol I, 7 (Sept), p 780

*Toxorhynchites argentotarsis*, Ludlow, 1906, *Can Ent*, Vol XXXVIII, (Novi), p 367

This is the only species of the genus which is generally common and widely distributed in India. It is of large size and there are conspicuous lateral tufts

on the terminal segments of the abdomen. The wing is from 8.5 to 9 mm in length.

A good deal of variation occurs in the colouration of the scales covering the proboscis, the sides of the mesonotum, and the prothoracic lobes, and also more especially in the leg markings. There is a much greater extent of white scaling on the tarsi in specimens from Bengal and Assam than in those from the Western parts of India, intermediate variations are, however, frequently met with. The names given as synonyms above appear to refer to forms of one and the same species. I have been able to consult nearly all the descriptions in the original and cannot discover reasons why any of them should be regarded as referring to distinct species.

Distinguishing characteristics, male and female —

The straight stout basal part of the proboscis purple or dark blue, usually some greenish scales at the flexure, palpi purple, those of the male with pale scaling at the apex of the 1st segment, and a subapical ring on the 2nd, more marked in some specimens than in others. Mesonotal scales dull bronzy-brown or greenish-brown, lighter towards the sides where they may be bluish or greenish, those over the wing roots and on the scutellum with a brighter sheen and either bronzy or bluish-green, scales covering the prothoracic lobes either brassy with bluish reflections, deep blue, or green, those on the proepimera usually white or silvery. Wings cross-veins *r-m* and *m-cu* much nearer the base of the wing than *s* (*vide* Text-figure A above). Abdomen 1st tergite blue or green in the middle, pale yellow at the sides, the 2nd to the 7th deep blue, deep green, violet, or purple, the 2nd, 3rd, and 5th with large lateral yellow patches, similar but much smaller markings on the 4th, lateral tufts of long hairs on segments 6 to 8, those on 6 partly pale yellow partly black, on 7 black, and on 8 orange, some flat blue scales overlapping the base of each tuft, 4th sternite mainly dark, the 5th and 6th yellow, 7th dark bluish in the middle and basally, with lateral yellow patches. In the male the abdomen is usually darker dorsally than in the female, the lateral yellow patches being smaller.

Variation in the tarsal markings of the female. In one specimen of apparently this species from the Andamans the fore tarsi are entirely dark, this may be an unusual aberration. In all the other specimens examined (47, including one other from the Andamans) the 1st segment is mainly white, there is usually a narrow basal dark ring, and in some specimens there is also an apical dark ring, but more often the segment is white from near the base to the tip, there is a basal white ring on the 2nd segment varying in size from about  $\frac{1}{4}$  to  $\frac{3}{4}$  or more of the length of the segment, the 3rd to the 5th segments are always dark. On the mid tarsi the variation is very considerable, in the darkest form there is a subbasal white ring on the 1st segment and a basal white ring on the 2nd occupying rather more than half the length, and in the lightest form the five segments are white except for a dorsal dark mark at the base of the 1st and a dark tip to the 5th, between these two extremes numerous intermediate variations occur. On the hind tarsi the 1st segment may be entirely dark or there may be yellowish or white scaling

forming a more or less complete basal or subbasal ring, there is always some pale scaling on the 2nd segment which may form a narrow subbasal ring but more often the larger part of the segment is white, the last three segments are always dark

The tarsal markings of the male from an examination of 36 males from various parts of India it appears that there is much less variation in the leg markings than in the female. The fore tarsi are often entirely dark but in some specimens there is some light bluish scaling at the base of the 1st segment on the underside, and also sometimes at the base of the 2nd, in other specimens the bluish scaling is replaced by white, but even in the extreme forms these markings do not form very definite rings. On the mid tarsi there are usually well-defined white markings near the bases of the 1st and 2nd segments, and in many specimens from Bengal and Assam there is a wide subbasal ring on the 1st occupying about half the segment, and a basal ring on the 2nd covering about two-thirds. On the hind legs the 1st segment may be entirely dark, or there may be some pale scaling at the base not forming a very definite ring, on the 2nd segment there is always a basal or subbasal white ring occupying half or more of the length, segments 3 to 5 are always dark on the mid and hind legs

Parts of the male genitalia are illustrated on one of the accompanying Plates, Figs 11 to 17

Specimens have been examined from the following places —

Bombay Presidency —North Kanara, Karwar, Kadra, and Yellapur, ix and x 1921 (*Barraud*), bred from larvae found in tree-holes, bamboo stumps, and earthenware jars

Bengal —Eastern Bengal, Chittagong, viii 1922 (*Barraud*), North Bengal, Sukna, x 1922 (*Barraud*)

Assam —Nongpoh, Hailong, and Dibrugarh, vii and viii 1922 (*Barraud*), Nowgong (*James John* collr)

Eastern Himalayas —Tindharia and Sureil, x 1922 (*Barraud*)

Burma —Thayetmyo (*W J S Ingram*), Katha, vii 1923 (*Major Taylor*)

Andaman Islands —vii 1926 (*Major Covell*)

### ***Megarhinus quasifer* (Leic)**

*Teromyia quasifer* v, Leicester, 1908, *Cul Malaya*, p 51

*Toxorhynchites javaensis*, Theobald, 1911, *Tyde v Ent*, Vol LIV, p 233

I have not seen any specimens of this species. It evidently resembles *M splendens* (Wied.) closely but differs in the characters given in the synoptic table, and possibly in other small details. It is mentioned as occurring in Sikkim in Edwards' Synopsis published in Vol X, of this *Journal*, p 459. The type specimens are in the British Museum, from the Malay Peninsula (*Leicester*). A good description will be found in Leicester's work cited above.

## ORIENTAL GENERA AT PRESENT UNREPRESENTED IN INDIA

The only Oriental genera of which no species have yet been found in India are *Zeugomyia* Leic , *Wyeomyia* Theo , and *Pardomyia* Theo , these have not therefore been dealt with in this Revision One species of the first is known from the Malay Peninsula, one of the second from the Philippine Islands, and two of the last, one from Malay and the other from the Philippines It is possible that, with further collecting representatives of one or more of these genera may be found to exist in the Indian sub-continent





#### EXPLANATION OF PLATE XIV.

Camera lucida drawings illustrating the structure of the male genitalia in Indian species of *Heizmannia*. All the figures are drawn to the scale shown under Fig 10

- |       |                       |  |    |
|-------|-----------------------|--|----|
| Fig 1 | <i>H himalayensis</i> | Clasper  |    |
| " 2   | <i>H indica</i>       | Do   |    |
| " 3   | <i>H chandi</i>       | Do   |    |
| " 4   | <i>H metallica</i>    | Do   |    |
| " 5   | <i>H chandi</i>       | Process on inner surface of side-piece ('harpago') |    |
| " 6   | <i>H indica</i>       | Do   | Do |
| " 7   | <i>H covelli</i> sp n | Do   | Do |
| " 8   | <i>H metallica</i>    | Do   | Do |
| " 9   | <i>H himalayensis</i> | Do   | Do |
| " 10  | <i>H covelli</i> sp n | Side-piece showing hair-tufts, clasper, etc        |    |

Figs 5 to 9 are of very transparent structures and were drawn from heavily stained preparations

## EXPLANATION OF PLATE XV.

Camera lucida drawings illustrating the structure of the male genitalia in Indian species of *Megarrhinus*. All the figures are drawn to the scale shown under Fig 21

Fig 11 *M. splendens* Ventral view of one side of the hypopygium, ninth segment and hairs, etc., of side-piece not shown

Lettering —

S Side-piece

C Clasper

AC Appendage of clasper

BL Basal lobe seen through side-piece

HF Harpaginal fold

BM Basal membrane

EXA External apodeme

BA Basal apodeme

PP Parameral plate

PH Lateral plates of phallosome

AS Chitinization of anal segment

CR Crown of ditto

„ 12 *M. splendens* Basal apodeme

„ 13 „ Parameral plate

„ 14 „ Chitinizations of anal segment

„ 15 „ Basal lobe and harpaginal fold

„ 16 „ Lateral plate of phallosome

„ 17 „ Ninth tergite

„ 18 *M. kempi* Ninth tergite

„ 19 „ Clasper

„ 20 „ Lateral plate of phallosome

„ 21 „ Ninth sternite

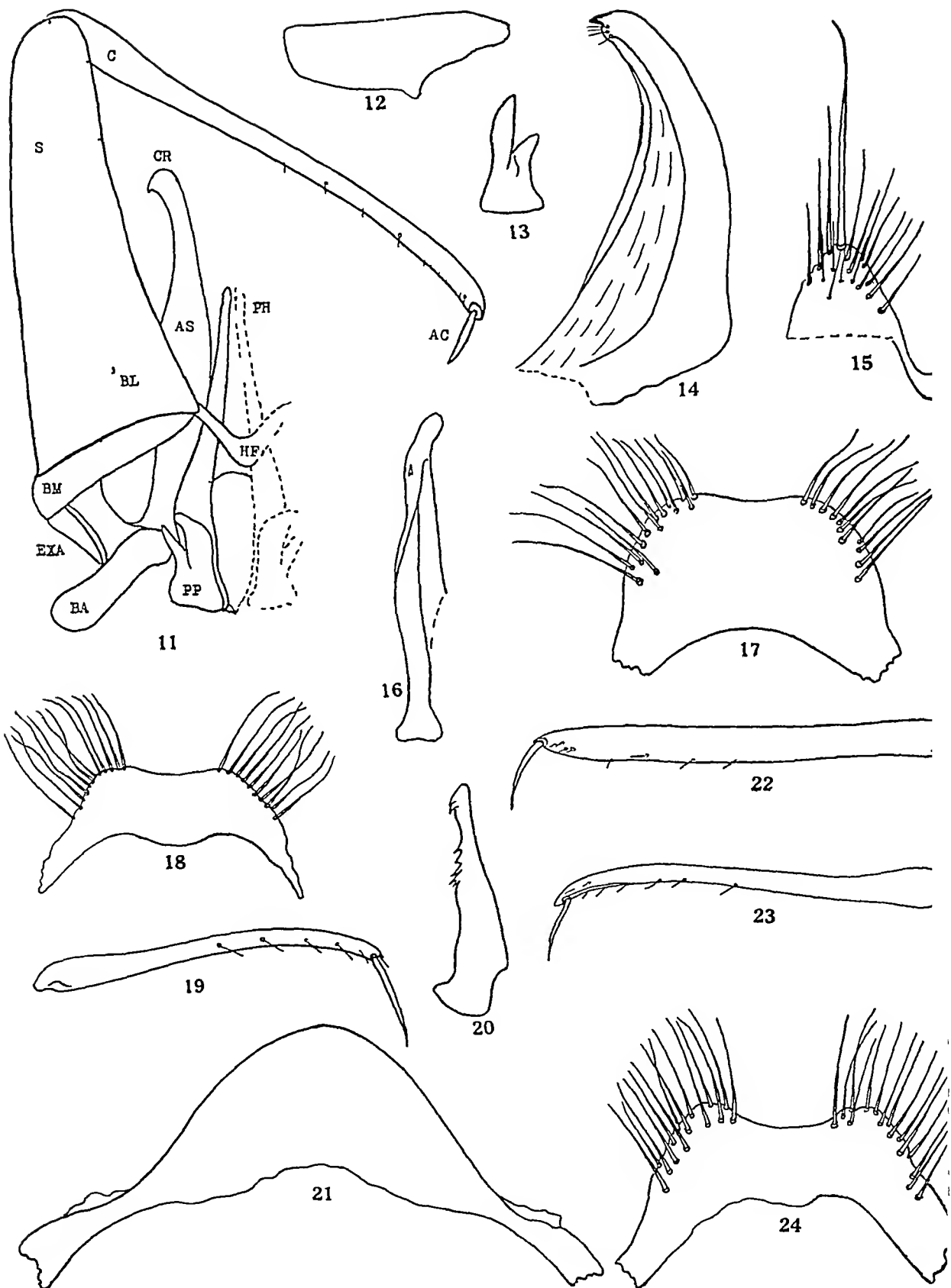
„ 22 *M. graveleyi* Clasper

„ 23 „ Clasper

„ 24 „ Ninth tergite

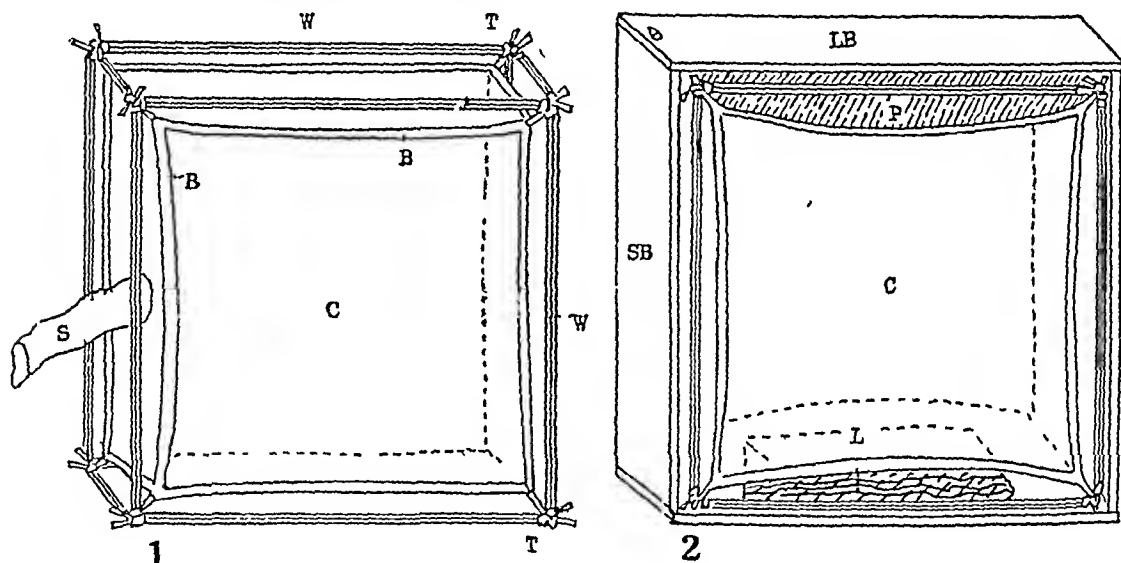
Figs 12 to 24 are drawn from flat preparations





would be likely to become entangled and crushed. A wooden box is made to contain the cage and should be just large enough to allow of the latter being easily inserted and withdrawn without risk of damaging the netting.

When packing live mosquitoes for transmission by train, motor, or parcel post, they are introduced into the cage through the sleeve and the latter then secured with tape. A folded pad of wet lint is first placed at the bottom of the box, the pad being sufficiently thick to press against the bottom of the mosquito netting when the cage is pushed down into the box over it. Some raisins are placed on the top of the cage, on which the mosquitoes may feed, the raisins being kept in place by a thick pad of cotton wool filling up the space between the top of the cage and the lid of the box (Fig 2). The lid is attached to the box with only one screw so that it can be turned to one side and the cage easily removed without the necessity of withdrawing nails and screws. The lid is firmly secured



to the box also with strong tape or webbing, and the whole then done up in brown paper and tied with string.

#### EXPLANATION OF TEXT-FIGURES

- 1 Wire frame and cage, showing method of securing latter inside former
- 2 Method of packing cage in box (on side of box removed to show interior)

Lettering —W, wires forming frame, T, tapes fastening wires of frame to one another and securing cage to frame, C, cage made of small mesh mosquito netting, S, sleeve, B, tape binding to edges of cage, P, cotton wool pad filling space between lid of box and top of cage, holding in place some raisins resting on mosquito netting, LB, lid of box, SB, side of box, L, pad of wet lint lying on bottom of box and sufficiently thick to press against mosquito netting forming bottom of cage.

The apparatus described above is quite simple and can be made in the laboratory, or in almost any Indian bazaar, at very small cost.

## Details of experiments carried out with the apparatus described in this paper

Serial number of experiment	Number of mos- quitos used	Sent by post from, and date	Place sent to	Date box opened	Number of mos- quitos found alive	Number of days enclosed in cage and box	Approximate distance travelled in miles	REMARKS
1	10 female <i>Anophelis</i>	Karnal, Punjab 17-7-1928	Kasauli	18-7-1928	9	1	100	
1 (R)	9 female <i>Anophelis</i>	Kasauli 18-7-1928	Karnal	20-7-1928	8	3	200	Same specimens used as in 1. Lint redamped before return. Eight mosquitoes survived double journey.
2	10 female <i>Anophelis</i>	Karnal 7-8-1928	Bombay	10-8-1928	8	3	1,035	
2 (A)	10 female <i>Culex</i>	"	"	10-8-1928	10	3	1,035	
3	10 female <i>Anophelis</i>	Karnal 23-11-1928	Coonoor, Nilgiri Hills	28-11-1928	10	5	1,940	
3 (A)	10 female <i>Culex</i>	"	"	28-11-1928	10	5	1,940	
3 (R)	10 female <i>Anophelis</i>	Coonoor 28-11-1928	Karnal	4-12-1928	8	11	3,880	Same specimens used as in 3. Lint redamped before return. Eight mosquitoes survived the double journey.
3 (A) (R)	10 female <i>Culex</i>	"	"	4-12-1928	10	11	3,880	
4	10 female <i>Anopheles</i>	Karnal 29-11-1928	Gauhati, Assam	5-12-1928	7	6	1,460	
4 (A)	10 female <i>Culex</i>	"	"	5-12-1928	10	6	1,460	
5*	26 female <i>Anopheles</i>	Gauhati, Assam 10-12-1928	Karnal	14-12-1928	19	4	1,460	
5 (A)*	14 female <i>Culex</i>	"	"	14-12-1928	14	4	1,460	
6	11 <i>Anopheles</i> males and females	Madras 12-3-1929	Kasauli	18-3-1929	6	6	1,754	The lint appeared almost dry when box was opened.
6 (A)	11 female <i>Culex</i>	"	"	18-3-1929	5	6	1,754	

\*Larvae were obtained from eggs laid by both *Anophelines* and *Culex* used in these experiments. Some of the mosquitoes lived for one month after they were received, at which date the experiments were terminated.

Of the total number of mosquitoes used in the experiments an average of over 80 per cent survived, and in the case of four batches there were no casualties in spite of the fact that in one case the mosquitoes travelled about 3,880 miles by parcel post

In addition to the experiments detailed above, the method described has recently been tested in a practical manner by Major J A Sinton, V C, O B E, I M S, Director, Malaria Survey of India, during a rapid tour through the Vizagapatam Agency On 15th March, thirteen caught mosquitoes were placed in a cage at Ambadola, and the box containing this was packed in a larger box The lint (placed in this case over the raisins) was damped again two days later and the box was then brought back by motor and train to Kasauli, a distance of about 1,260 miles On opening the box on 21st March all the mosquitoes were found to be alive, and the female *Anophelines*, eight in number, were dissected for the detection of malaria parasites Thirty-eight other mosquitoes, bred from larvae collected in various places, were placed in another cage at Titlagarh, Patna State, on 17th March The cage was packed up and brought to Kasauli, a distance of about 1,230 miles The box was opened on 21st March and thirty-six of the mosquitoes were found to be alive They were then repacked and posted to Karnal, a distance of about 100 miles from Kasauli, and when opened on the 22nd March, thirty-five of the mosquitoes were still alive It may be mentioned that during a considerable part of the journey from the Vizagapatam Agency, through Central India, to Kasauli, the maximum shade temperature was about 100°F, and the atmosphere very dry, in contrast to the conditions of humidity ruling in Bombay in August, when other experiments, referred to above, were made

#### SUGGESTED USES FOR THE METHOD DESCRIBED ABOVE

The method described will probably be found useful in many ways Some of the uses to which it could be put, which immediately suggest themselves, are as follows —

- 1 Collectors may be sent out from a Central Laboratory for three or four days journey to a selected area where malarial conditions are being studied, mosquitoes may be collected and brought back for dissection By the time the mosquitoes have arrived, they will have digested any blood in their alimentary tracts and be in a suitable condition for dissection

- 2 Freshly hatched *Anopheles* mosquitoes could be forwarded to a Central Laboratory, or other convenient centre, for feeding experiments in connection with malaria research, to test the susceptibility to infection of various species, with different strains and types of the parasite

- 3 Living gravid female mosquitoes could be sent by collectors, by parcel post, from various parts of the country for breeding purposes The method described has already proved of value in this direction When received such mosquitoes can be put up in a suitable manner for oviposition in the base laboratory, where the study of the early stages and life-history can be carried out

under the best conditions. This may also save a good deal of time and money in travelling about in the endeavour to obtain rare or local species for this purpose. The cost of sending one of the boxes by registered parcel post is about fourteen annas.

4. In connection with the experimental transmission of disease by mosquitoes, the method described may be found of value. The feeding of such insects upon infected human beings, or animals, is usually carried out in an area where the particular disease is endemic. It is desirable then to carry out the second part of the experiment, viz., the refeeding of the mosquitoes on healthy human volunteers or experimental animals, in a non-endemic area, thus removing the objection, nearly always present if the second part of the experiment be carried out in the endemic area, that the volunteers or animals may have contracted the disease by some means other than by the bites of the mosquitoes used in the experiments. By using the method described above the mosquitoes could be safely sent by train, car, or through the post, for any length of journey occupying from one to ten days, with a good chance of the majority surviving and being in suitable condition for the second part of the experiment. It may be mentioned that the risk of the mosquitoes escaping from the cage during transit is practically *nil*, if they are properly packed.

#### ACKNOWLEDGMENTS

In conclusion I wish to thank those who have kindly co-operated with me in the experiments: Major J. A. Sinton, V.C., O.B.E., I.M.S., Major G. Covell, I.M.S., Capt. H. W. Mulligan, I.M.S., of the Malaria Survey of India, Mr. C. S. Swaminath of the Kala-azar Commission, Gauhati, Assam, Major J. B. de W. Molony, O.B.E., I.M.S., D.A.D.H., Madras district, Bangalore and Coonoor, and the Director, King Institute, Madras.

*Postscript*—Since writing the above an attempt has been made to transport living mosquitoes from Karnal, Punjab to London. On April 4th, twenty-two mosquitoes were enclosed in a cage at Karnal. The box containing the cage was then done up in brown paper and string, wrapped in a dressing gown, and packed in a sack-shaped kit-bag. The box was not examined until my arrival in London on April 20th, when eleven mosquitoes were found to be alive. The species used in the experiment were *A. fuliginosus* 10 females (2 survived), *A. culicifacies* 1 female (died), *C. fatigans* 8 females (6 survived), *C. vishnu* 2 females (both survived), *Aedes (S.) aegypti* 1 female (survived). The journey occupied 16 days and is approximately 6,000 miles. On April 22nd the mosquitoes were handed to Dr. P. A. Buxton, London School of Tropical Medicine. By that date one of the two surviving *A. fuliginosus* was dead and the other moribund. The single *Aedes (S.) aegypti* was not kept as large numbers of the Karnal race had already been bred from the egg in London. It is hoped that eggs may be obtained from the survivors belonging to the two species of *Culex*.

During April and May this method of transporting live mosquitoes has been used successfully by officers of the Malaria Survey of India.

# EXPERIMENTS ON PRE-INFECTIONAL IMMUNIZATION AGAINST RABIES WITH CARBOLIZED AND ETHERIZED VACCINES

BY

LIEUT-COL. T H GLOSTER, I M S (Retd ),

W A BEER, I M D,

AND

SUR-ASSISTANT SURGRONS M RAMAN NAMBIAR AND

S SITARAMA SASTRY,

*Pasteur Institute of Southern India, Coonoor*

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CORNWALL and Beer (1926) proposed that certain standard conditions should be observed in testing the protective value of any form of anti-rabic vaccine. These are —

- (1) The animals should be from half to three-quarters grown
- (2) The weight of fixed virus used in the course of treatment should be strictly proportionate to the body-weights of the individual animals
- (3) The weight of fixed virus used for the animals should be strictly proportionate to the weight given or to be given to human patients

In experiments hitherto undertaken in India to determine the protective value of carbolized vaccine, conditions (2) and (3) have not been observed.

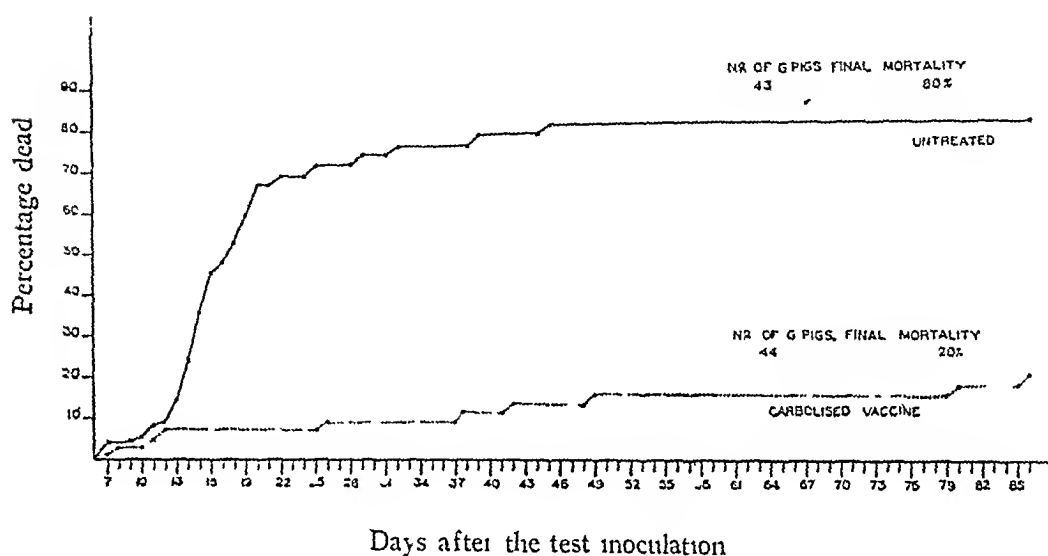
At the Pasteur Institute, Coonoor, the usual scheme of immunization in such experiments has been to inoculate guinea-pigs subcutaneously with from 0.5 cc to 1 cc of 1 per cent carbolized vaccine daily for 14 days irrespective of the weight of individual animals.

Stevenson, King and Lahiri (1926) employed the same method of immunization of guinea-pigs (0.5 cc of 1 per cent vaccine daily for 14 days) in their investigation on the effect of heat on the Kasauli carbolized vaccine.

Harvey and McKendrick in experiments with the same object gave their rabbits 5 cc of a 0.5 per cent carbolized vaccine daily for 14 days.

# CHART 1

Diagram to illustrate the effect of pre-infectional inoculation with carbolized fixed virus vaccine on guinea-pigs



King, Nicholas and Lahiri (1926) in a further series of experiments at Kasauli to test the effect of heat on the carbolized vaccine used rabbits and modified Harvey and McKendrick's method of immunization only to the extent of using a 0.6 per cent vaccine.

In one experiment they gave twice the quantity of vaccine to a small series of rabbits and, as they obtained better protection with the double dose, they concluded that the doses used in the main experiment were not maximal.

It is worth while to consider the degree of protection conferred by the relatively large quantities of carbolized vaccine given by these workers.

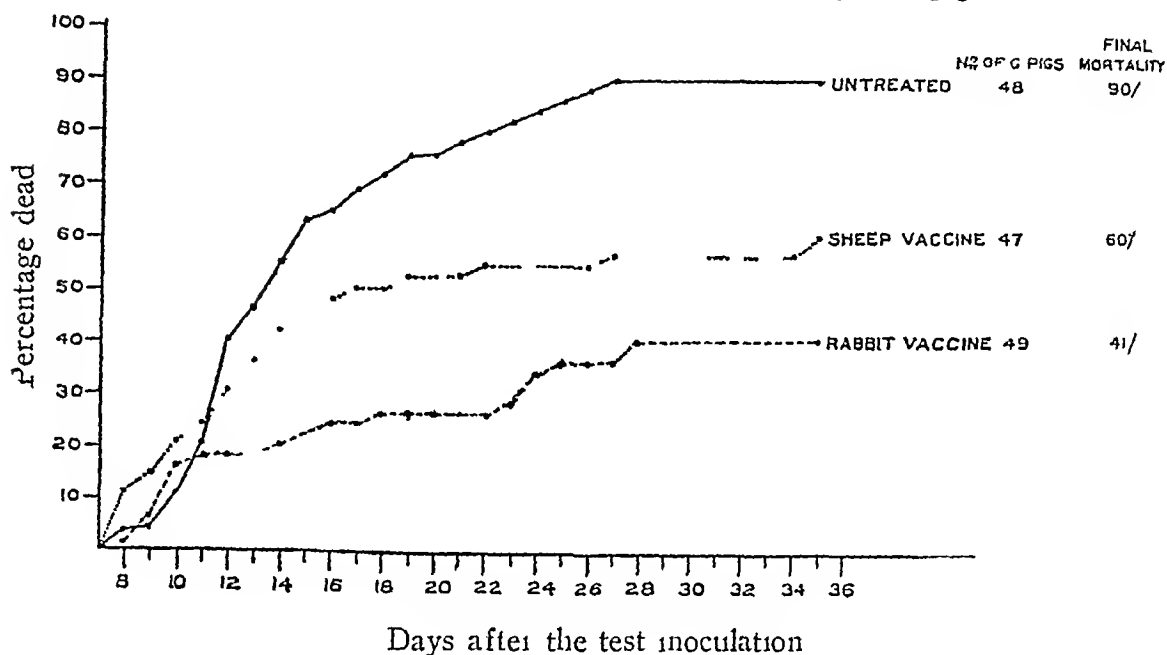
Cornwall (1916 unpublished) summarized the results obtained at Coonoor by immunizing guinea-pigs with fresh vaccine and vaccine kept in the refrigerator for 2 months,—the test dose being an intramuscular injection of street virus —

Of untreated guinea-pigs	80 per cent died of rabies
Of guinea-pigs treated with fresh vaccine	65 " " "
Of guinea-pigs treated with vaccine which had been kept in the refrigerator for 2 months	45 " " "

Stevenson, King and Lahiri (1926) found that their vaccinated guinea-pigs had acquired little, if any, immunity when tested by intramuscular injection of street virus, the rabies mortality among the vaccinated and control animals being respectively 83 per cent and 88.5 per cent.

CHART 2

Diagram to illustrate the effect of pre-infectional inoculation with carbolized rabbit and sheep fixed virus vaccine on guinea-pigs



Cunningham (unpublished experiment in 1927) failed to obtain any protection against a subsequent corneal infection with street virus by immunizing rabbits with carbolized vaccine in doses proportionate to their weight or even in doses 10 times as large

The following table gives a summary of the results obtained by the various workers referred to above —

The experiments detailed below were carried out at the Pasteur Institute, Coonoor, in 1927-28. Their objects were (1) to determine the immunizing power, if any, of carbolized vaccine when given in doses proportionate to those given to human beings at Coonoor, (2) to compare the immunizing power of carbolized vaccine in doses proportionate to those given to human beings at Coonoor with that of etherized vaccine in doses proportionate to those given to human beings by Alvisatos, (3) to compare the immunizing power of etherized vaccine given by Alvisatos' method in doses proportionate to weight with that of carbolized vaccine in the same doses given at the same intervals

The carbolized vaccine was the same as that used for human patients, viz, a 1 per cent suspension of fixed virus brain in normal saline containing 0.5 per cent of phenol

Before use it was diluted to 1 in 20 with carbolized saline. The dose given in experiments I, II and III was 0.16 c.c. of the 1/20 per cent suspension per 100 grammes weight of guinea-pig daily for 14 days. The total weight of brain substance injected was therefore 1.1 mg. per 100 grammes of weight which is



TABLE  
Showing results obtained by various experimenters with fresh carbolized fixed virus

	Animals used	NUMBER OF ANIMALS		Amount of fixed virus brain given per kilo of body-weight	Test of immunity	Interval between last immunizing dose and test dose	PERCENTAGE OF DEATHS FROM LABIES	
		Treated	Untreated				Treated	Untreated
Harvey and McKendrick (1922)	Rabbits	48	49	175 to 350 mg	Street virus into neck muscles	Not stated	65.3	97.9
King, Nichols and Lahiri (1926)	Do	21	21	281 mg	Fixed virus into neck muscles	5 days	43	76
Do	Do	11	21	560 mg	Fixed virus into neck muscles	5 days	18	76
Gloster and Taylor (1926)	Do	30	30	230 mg	Street virus into neck muscles	15 to 31 days	0	40
Do	Do	24	24	165 mg	Street virus on scarified cornea	7 days	50	100
Cruckshank (1923)	Do	31	11	156 mg	Street virus into thigh muscles	25 days	32.3	100
Stevenson, King and Lahiri	Do	35	35	144 mg	Street virus into thigh muscles	19 to 24 days	83	88
Harvey and Acton (1923)	Monkeys	15	19	770 mg	Street virus into neck muscles	Not stated	80	94.7
Acton and Knowles (1923)	Do	20	13	100 mg	Street virus into neck muscles	Not stated	55	84.6
Gloster Beer Nambiar and Sastri (1927-28)	Guinea-pigs	117	116	11.2 mg	Street virus into neck muscles	17 to 25 days	30.7	79.3

proportionate to the amount given at Coonoor to adult patients, assuming the weight of an average adult to be 63.6 kilograms

The etherized vaccine was prepared as follows —

Rabbits' fixed virus brains were removed aseptically and suspended in 60 c c of Merck's ether in sterile bottles. In experiment III whole brains were

CHART 3

Diagram to illustrate the effect of pre-infectional inoculation with carbolized and etherized fixed virus vaccines on guinea-pigs

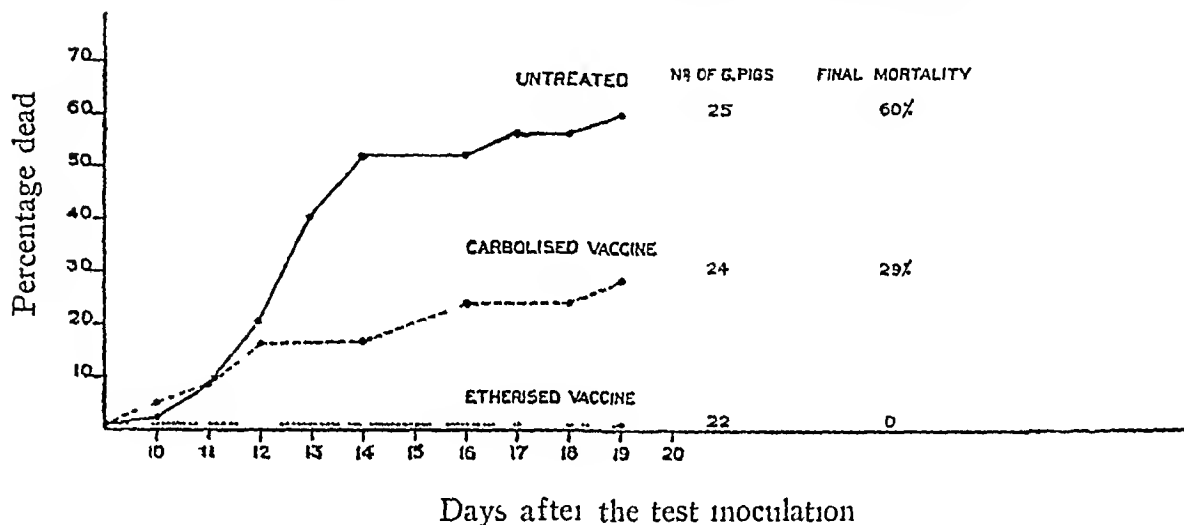
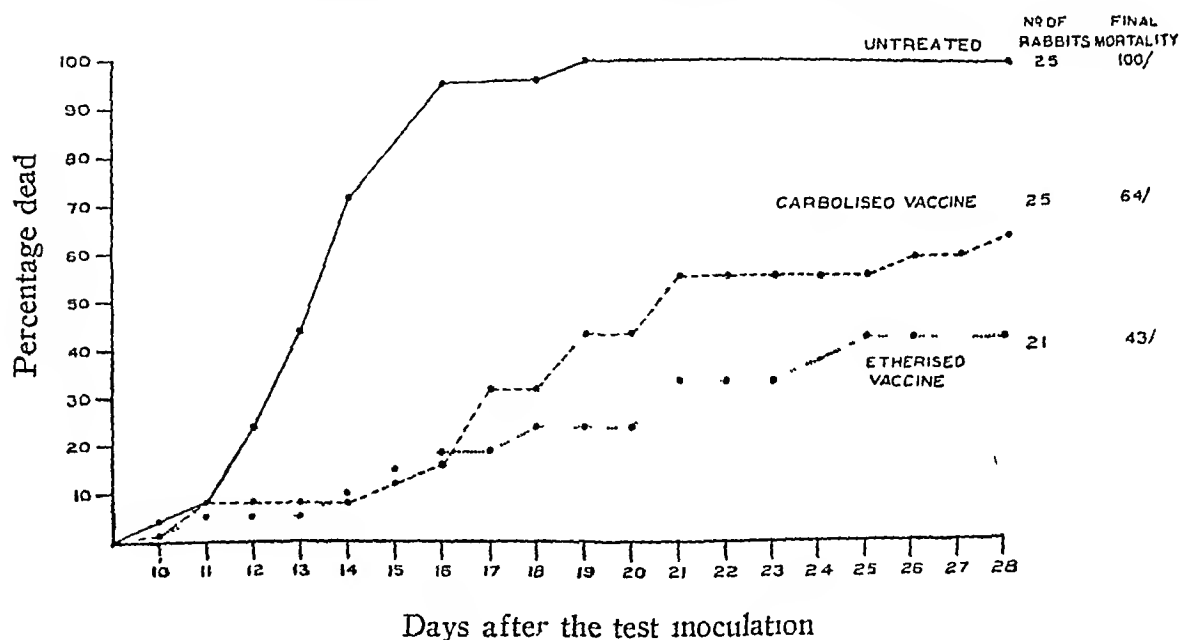


CHART 4

Diagram to illustrate the effect of pre-infectional inoculation with carbolized and etherized fixed virus vaccines on rabbits.



immersed in the ether and in experiment IV half brain. After the required period of immersion the brains were removed and cut up into several pieces in a sterile glass dish which was then put under a Bulloch's jar for one hour, the air being exhausted 3 or 4 times during this period to remove the ether. The brain substance was then emulsified in 2 parts of sterile neutral Merck's glycerine containing 25 per cent of normal saline. The emulsion was kept in the refrigerator (temperature  $40^{\circ}$ — $42^{\circ}$ F), and dilutions were made as required for the immunizing injections.

The longest period for which the glycerine emulsion used for preparing the injections remained in the refrigerator before use was 72 hours. All the etherized brains used in the immunization experiments were tested for infectivity by inoculating 0.002 grammes of the brain substance under the dura mater of rabbits or guinea-pigs. None of the animals developed rabies showing that we were dealing with a killed or highly attenuated virus. The etherized vaccine was given according to Alvisatos' scheme, doses for weight, as shown below—

*Doses per 100 grammes weight of guinea-pig or rabbit*

1st day—25 mg (0.125 c.c. of 2 per cent suspension of fixed virus brain kept in ether for 84 or 72 hours)

2nd day—25 mg (0.125 c.c. of 2 per cent suspension of fixed virus brain kept in ether for 84 or 72 hours)

5th day—25 mg (0.125 c.c. of 2 per cent suspension of fixed virus brain kept in ether for 72 or 60 hours)

7th day—15 mg (0.075 c.c. of 2 per cent suspension of fixed virus brain kept in ether for 72 or 60 hours)

9th day—15 mg (0.075 c.c. of 2 per cent suspension of fixed virus brain kept in ether for 72 or 60 hours)

12th day—15 mg (0.075 c.c. of 2 per cent suspension of fixed virus brain kept in ether for 60 or 48 hours)

14th day—10 mg (0.05 c.c. of 2 per cent suspension of fixed virus brain kept in ether for 60 or 48 hours)

15th day—10 mg (0.05 c.c. of 2 per cent suspension of fixed virus brain kept in ether for 60 or 48 hours)

Total 14 mg of etherized fixed virus brain in 15 days

In experiment IV the carbolyzed and etherized vaccine were prepared from the same fixed virus brains, one-half of each brain being used for preparing the etherized and the other half for the carbolyzed vaccine. The carbolyzed vaccine was given in the same doses, for weight, of fixed virus brain substance and on the same days as the etherized vaccine.

In experiments I, II and III the animals were kept under observation for 3 months from date of receiving the test dose. In experiment IV the observation period was 2 months from date of receiving test dose.

*Experiment I*

Immunizing effect of small doses of carbolized fixed virus The doses of vaccine were proportionate to those given to patients Guinea-pigs were used in the experiment —

(1) Groups	Immunized	Controls
(2) Initial number	50	50
(3) Deaths before test dose	2	
(4) Number given test dose	48	50
(5) Average weight in grammes	584	560
(6) Deaths at test dose		
(7) Deaths after test dose not due to rabies	4	7
(8) Final comparative strength	44	43
(9) Deaths from rabies in (8)	9	34
(10) Proportion of deaths from rabies (9) and (8)	0.2	0.8
(11) Average period in days between infection and death in (9)	40	20

The immunized animals were given 14 daily injections of carbolized vaccine prepared from fixed virus rabbit brains and cords The dose was 0.16 c.c. of 1/20 per cent suspension per 100 grammes Test dose was 1 c.c. of a 1/40 suspension of guinea-pig street virus (1st passage from dog) per 500 grammes of body-weight, injected into the muscles of neck—25 days after the last immunizing dose The incubation period of the street virus for the guinea-pig after subdural injection was 13 days

*Experiment II*

Immunizing effect of doses proportionate to those given to human beings of carbolized rabbit fixed virus and carbolized sheep fixed virus compared Guinea-pigs were used in the experiment —

(1) Groups	Carbolized rabbit fixed virus vaccine	Carbolized sheep fixed virus vaccine	Controls
(2) Initial number of animals	50	50	50
(3) Deaths before test dose			1
(4) Number given test dose	50	50	49
(5) Average weight in grammes	584	596	526
(6) Deaths at test dose		1	
(7) Deaths after test dose not due to rabies	1	2	1
(8) Final comparative strength	49	47	48
(9) Deaths from rabies in (8)	20	28	43
(10) Proportion of deaths from rabies (9) and (8)	0.41	0.6	0.9
(11) Average period in days between infection and death in (9)	15.2	19	14.6

The test dose was given 17 days after the last dose of vaccine. It was 1 c.c. of a 1/60 suspension of guinea-pig street virus (1st passage from dog) per 500 grammes and was injected deeply into muscles of neck. Incubation for 1st passage guinea-pig 17 days after intramuscular inoculation.

### *Experiment III*

Guinea-pigs were used in the experiment —

	Etherized vaccine, Alvisatos' method doses proportionate to those used for human beings	Carbolized vaccine, Indran method doses proportionate to those used for human beings	Controls
(1) Initial number	25	25	25
(2) Deaths before test dose			
(3) Number given test dose	25	25	25
(4) Average weight in grammes	562	546	614
(5) Deaths at test dose	2*	1*	
(6) Deaths after the test dose not due to rabies	1		
(7) Final comparative strength	22	24	25
(8) Deaths from rabies in (7)	0	7	15
(9) Proportion of deaths from rabies (7) and (8)	0/22 0.0	7/24 0.29	15/25 0.6
(10) Average period in days between infection and death		11.4	11.0

\*Due to spinal injury at time of giving the test dose

The test dose was a 1/50 suspension of 5 different street virus guinea-pigs' brains (1st passage from dogs). One c.c. of this suspension was injected deeply into the muscles of the neck 17 days after the completion of immunization.

The incubation periods of the 5 street viruses after subdural inoculation into guinea-pigs varied from 11 to 16 days.

### *Experiment IV*

Rabbits were used in the experiment —

	Carbolized fixed virus	Etherized fixed virus	Controls
(1) Initial number	25	25	25
(2) Deaths before test dose	0	2	0
(3) Number given test dose	25	23	25
(4) Average weight in grammes when given test dose	1,775	1,717	1,494
(5) Deaths at test dose			
(6) Deaths after test dose not due to rabies		2	
(7) Final comparative strength	25	21	25
(8) Deaths from rabies in (7)	16	9	25
(9) Proportion of deaths from rabies (7) and (8)	16/25 0.64	9/21 0.43	25/25 1.00
(10) Average period in days between infection and death	18.4	18.3	13.8

The carbolized and etherized vaccines were prepared from the same brains, half of the brain being made up into carbolized vaccine and the other half put in ether. The half brains were kept in ether for 48, 60 and 72 hours. The rabbits were given etherized vaccine according to Alivisatos' scheme, doses for weight, 72 hours brain being used for the first 2 injections, 60 hours brain for the next three and 48 hours brain for the last three.

The carbolized vaccine was given at the same time and in doses containing the same amount of brain substance for weight as in the corresponding doses of etherized vaccine.

The test dose was fresh guinea-pig street virus brain (1st passage from dog) incubation period 9 days for the guinea-pig after subdural injection. A thick emulsion was rubbed into the scarified cornea 12 days after the last immunizing injection.

#### METHOD OF TESTING IMMUNITY

Preliminary experiments at Coonoor had shown that, whereas 100 per cent of rabbits can be infected by inoculating street virus on the scarified cornea, only about 20 per cent of guinea-pigs could be infected in this way.

In view of these results the method of infection adopted in experiments I, II and III, for which guinea-pigs were used, was the intramuscular injection of street virus. The street virus was guinea-pig's brain, the animals being infected directly by subdural inoculation of the rabid dog's brain.

Suspensions of brain diluted from 1 in 40 to 1 in 60 were injected deeply into the neck muscles, the dose being 1 c.c. per 500 grammes weight of guinea-pig.

In experiment IV for which rabbits were used the method of infection was inoculation of a thick suspension of guinea-pig street virus (1st passage from dogs) on the scarified cornea.

Further details are given in the protocols and charts of the experiments.

#### DISCUSSION

In three separate experiments the immunity of guinea-pigs which had been treated with carbolized fixed virus in doses for weight proportionate to those used in the treatment of human beings was tested by the intramuscular injection of street virus from 17 to 25 days after the last immunizing dose. The summarized results of the three experiments show 36 deaths from rabies among 117 immunized animals and 92 deaths from rabies among the 116 controls, that is, rabies mortalities of 30.7 per cent and 79.3 per cent respectively.

The results compare favourably with those obtained at Coonoor and elsewhere in India by immunizing guinea-pigs with quantities of carbolized fixed virus from 19 to 30 times as large (*vide* Table). Cunningham (1927) failed to raise any immunity in rabbits with doses of carbolized vaccine proportionate to those used for human beings or even with doses 10 times as great.

Cunningham, as stated, used rabbits in his experiments and employed a severer test for immunity, viz, corneal inoculation of street virus, which gave 100 per cent rabies mortality among his controls. So our results are not comparable with his.

In two experiments etherized and carbolized vaccines were compared. In experiment III in which guinea-pigs were used the carbolized vaccine was given in doses proportionate to those given in India to human beings and the etherized vaccine in doses proportionate to those given to human beings by Alvisatos. The rabies mortalities among the etherized vaccine, carbolized vaccine and untreated groups were respectively nil, 29 per cent and 60 per cent. The test in this experiment was the intramuscular injection of a suspension of 5 different street viruses. It is seen that with a comparatively mild infection (60 per cent mortality among controls), pre-infectional immunization with etherized vaccine completely abolished the rabies mortality.

In experiment IV in which rabbits were used the carbolized and etherized vaccines were prepared from the same fixed virus brains. The doses of both vaccines contained the same amounts of fixed virus brain (per 100 grammes of rabbit) and were given at the same intervals. The test for immunity was the inoculation of street virus on the scarified cornea.

The rabies mortalities in the etherized vaccine, carbolized vaccine and control series were 43, 64, and 100 per cent respectively. The result suggests that weight for weight the etherized fixed virus has a higher antigenic value than carbolized fixed virus. On the other hand, a reference to the protocol of experiment IV shows that the carbolized fixed virus was somewhat less injurious than the etherized fixed virus.

The etherized fixed virus used in experiments III and IV was dead or highly attenuated as it failed to infect guinea-pigs or rabbits when injected subdurally.

### SUMMARY

(1) Carbolized vaccine in doses proportionate to those used for human beings confers considerable protection to guinea-pigs against subsequent infection with rabies.

(2) Guinea-pigs and rabbits immunized with etherized fixed virus by Alvisatos' method show a higher degree of immunity than is obtainable by immunization with carbolized fixed virus even when the latter is given in the same quantity for weight as the etherized fixed virus.

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**REPORT NO. I.****A RAT-FLEA SURVEY OF HOSUR, SALEM DISTRICT**

BY

P V SEETHARAMA IYER, M A

TOWARDS the end of August, an investigation unit of the King Institute, under me was sent to Hosur where it was soon joined by Doctors N Natarajan, M B, B S Sc, and P V George, B A., L M S, working under the Research Fund Association. My thanks are due to them for their help and to Col King for advice and guidance in the Survey and help in the report.

**GENERAL**

*Situation*—Hosur, a small town, is the head-quarters of the Hosur Taluk in the Salem District. It is 25 miles from Bangalore lying on the trunk road from Bangalore to Krishnagiri. The Morappur-Hosur narrow gauge railway connects Hosur with the main line of the South Indian Railway. Malur on the Madras and Southern Maharatta Railway is another railway station accessible to and used by the people of the Taluk. It was once a place of military importance and commands all lines of communication to Mysore.

*Hosur*—The town consists of two parts (1) Hosur proper or the new 'Peta' and (2) Therpet or the old 'Peta'. There are more than 1,000 houses in the town and the population according to the last census (1921) is 5,519.

The whole town presents a desolated appearance and a number of houses are seen all round abandoned and in ruins. The yearly visitation of plague has been mainly instrumental in the rapid decline of the town.

Therpet village lies south-east of the town at the foot of the Pagoda Hill. It was a place of importance but is now almost deserted. The streets are lined with abandoned choultries for pilgrims, but the few houses that remain are seen clustered round the village tank.

There are a number of small wayside hamlets lying scattered all round Hosur along the various routes of communication. A hamlet on the side of each of the main roads was also surveyed during the period (*vide* Maps).

**POPULATION**

The population according to the last census (1921) is 5,519. Due to chronic poverty and disease, the population figures show a steady decline.

**TABLE I**

Name of place		Number of houses	Population
1	Hosur town including Therpet	1,152	5,519
2	Pedda Jibi	50	250
3	Basti	16	80
4	Chanasundaram	12	60
5	Dinnur	17	70
6	Alasanattam	18	100



HOUSE TOWN  
 Showing Houses from which Rats were caught  
 (The Black Dots Indicate the Houses)

Showing Houses from which Rats were caught  
(The Black Dots Indicate the Houses)



A hand-drawn map of the area around Bangalore, showing roads, landmarks, and districts. The map includes labels for Bangalore, TOLL GATE, JIBI, PEDHA JIBI, DINNUR, R S, THERPET, CHANASANDRAM, SULESANDRAM, ALASANATHAM, POHOSUR, TOLL GATE BASTI, and Krishnagiri. It also shows the districts of Channarayana, Channarayana, and Channarayana. A north arrow is present in the top right corner.

## ECONOMIC CONDITION OF THE PEOPLE.

The grain trade is concentrated in the hands of 'Komatties' who carry on a brisk trade with the Mysore border. The chief centres of distribution and exchange of different commodities are mainly the bazaar and the weekly market. Every Wednesday, at the weekly market, the ryot disposes of his produce and purchases the necessaries of life, such as cloth, salt, chillies, etc. Next in

importance is the trade in cattle and during the busy agricultural season, nearly a thousand head of cattle change hands on the weekly shandy day, the chief buyers being from the East Coast districts and Mysore

### HOUSING CONDITIONS

The houses are mostly tiled or thatched and usually consist of a single floor. The thatching material used for huts is mainly 'kambu' straw and 'cholam' stalks. The poor classes live in one room tenements but most of the houses have at least two rooms, occasionally with a court-yard in front. The houses are very insular, the cattle of the house and the inmates all usually being accommodated in the same room. The room serves as a sleeping room, the floor either bare or often with a grass mat serving as a bed. One can observe scattered all round the room wooden boxes, heaps of firewood, and dung cakes used as fuel, which give ideal shelter for rats.

The majority of the houses have mud walls and these are frequently riddled with rat holes and crevices. 'Rigi' is stored in pits, excavated in the floor of the houses. These pits are 10 feet to 12 feet deep and have a flat bottom and a dome-like top with a manhole. The common type of floor made of beaten earth offers no obstacle to rats which form extensive burrows.

The roofs of houses consist of wooden beams supporting a lattice of bamboos, on which are laid two or three layers of country tiles with deep channels. These tiles afford great facilities for the sheltering of rats during the day time.

Every house has, either in front or behind, an open drain into which is thrown all the refuse from the house. It is surprising to see people in this plague infected area regarding the presence of rats in the houses with tolerance and even reverence. Some Hindus regard rat destruction as irreligious and refuse permission to have traps placed in their houses.

### METEOROLOGICAL CONDITIONS

*General*—The town is situated at an elevation of about 2,875 feet above sea-level, the Pagoda Hill in the vicinity being 3,126 feet. The climate is salubrious and pleasant through the greater part of the year. Exact observations regarding humidity are not available. During the survey period a few records were made of the daily fluctuations in the state of the atmosphere. The maximum and minimum wet and dry bulb temperatures reached were as follows—

TABLE II

Time	DRY BULB		WET BULB	
	Maximum	Minimum	Maximum	Minimum
8 a.m.	73.4	70.7	67.3	65.3
2 p.m.	83.8	78.8	71.6	66.5
8 p.m.	83.1	77.9	70.1	68.4

The relative humidity of the atmosphere was as high as 70 per cent in the mornings (8 A.M.) and during the day it gradually fell to 48.6 (2 P.M.) A steady rise was again noticed during the night time.

*Rainfall*—The distribution of rainfall is uneven. Rain sets in late in April and in a normal year heavy rains may be expected in May. Irregular showers occur in June, July and early August, the real monsoon rains falling during September and October. The average annual rainfall for the last nine years is 27.02 inches, the months of September and October contributing 9.57 inches on an average. Thus in 1925, more or less a typical year, the monthly rainfall was as follows: January nil, February nil, March, 0.26, April, 1.79, May, 7.69, June, 1.99, July, 0.81, August, 3.78, September, 6.76, October, 1.13, November, 2.8, December, 2.23.

The month of September this year—the period of the present survey—had been unusual in that it recorded the lowest rainfall for that month during the last nine years.

*Temperature*—The temperature is most equable. No official records of the temperature were available, but it is probable that the temperature rarely goes higher than 90°F.

During September which covers the period of survey, the maximum temperature recorded was only 83.8°F, while the minimum was 70.7°F. The hottest part of the year is generally in May and June.

### SURVEY

The period during which the survey was carried out extended from the 30th August to 2nd October, 1928. September is usually the beginning of the plague season in the locality. During September this year (1928), 14 attacks and 4 deaths from plague were recorded. Trapping of rats was done on 27 occasions.

### PROCEDURE ADOPTED

The traps used were of two kinds—one the usual wire caged trap allowing only ingress of rats through a hinged and weighted platform but with a single compartment, while the other, a bigger pattern, had a second compartment in addition.

Traps were distributed every evening in the areas selected and care was taken to see that traps were placed in portions of houses frequented by rats. In the early morning the cages were gathered and each trap containing rats was at once covered with a strong calico bag to prevent escape of any flea on the way to the laboratory. The bags with the traps were placed inside a wooden air-tight box and chloroform was added. In about 20 minutes the rats and the fleas on them were invariably found dead. Each cage was then taken on to a table with a sheet of white cloth spread over it and any loose fleas in the cage were gathered first. The dead fleas on the rats were combed on to the white surface and picked up with a fine camel hair brush moistened in alcohol. The majority of the dead fleas were in the bag and these were collected last.

Care was taken to collect all the fleas on every rat trapped. If more than one rat was present in a cage, the fleas were considered as coming equally from all the rats.

When the day's collection of rats had been dealt with, the fleas gathered from each cage were cleared in a boiling solution of caustic potash, for examination.

The following details were recorded regarding each rat —

- 1 Locality and the house in which it was trapped
- 2 Kind of rat and sex
- 3 Number of fleas gathered
- 4 Kinds of fleas and their sexes
- 5 Other ectoparasites found

#### DEFINITION OF ZONES

Hosur proper was divided for the purposes of the survey into 8 wards. The main roads passing through the heart of the town made it easy to effect a clear cut division into distinct areas.

Therpet forming part of Hosur town and a few other villages were also surveyed. These villages lie along the main roads and consist mostly of a cluster of thatched huts occupied by the poorest class of people depending on agriculture for their livelihood.

*Ward I High School Road*—This commences at one end of the town and runs straight passing through the bazaar area in its middle. The Mohammedans occupy the eastern half of the street exclusively, while the other half is mainly Hindu.

*Ward II Post Office Road*—Part of the trunk road running from Bangalore to Krishnagiri and contains the grain godowns and the cloth bazaar.

*Ward III Bazaar area*—Comprises the portion of the road cutting across the town from Malur to Matugiri. The bazaar proper comes within the area.

*Ward IV Janappar area*—Constitutes the northern portion of the town to the west of the Malur Road and to the north of the Post Office Road. This contains houses mostly belonging to labourers.

*Ward V Weaver area*—Mainly consisting of weavers, 'Pallis' and others trading in fish. It lies to the east of Malur Road and north of Post Office Road. This portion is cut up by a number of narrow lanes traversing it in all directions. The houses belong to the middle class people but are low roofed and ill-ventilated.

*Ward VI Vanyar area*—The area to the south of the High School Road and to the west of the Malur Road is mainly occupied by Vanyars who carry on a brisk trade in gingelly oil. Gollars, a caste of cattle graziers, also live here.

*Ward VII 'Hindu' area*—Mainly occupied by Hindus. The part of the town to the south of the High School Road and to the east of Malur Road comes under this ward.

*Ward VIII Taluk Office area*—This comprises the area covered by the Government buildings, Travellers' Bungalow, Police Lines, etc., and lies to the south of the town at a distance of about 2 furlongs

#### RESULTS OF THE SURVEY

*Distribution of rats*—During the survey, 379 rats were trapped. Barring a few houses, every house in each area was given a trap and sometimes two, and Table III (a) and (b) gives an idea of the rat prevalence in the various wards of the town and in the villages around

TABLE III (a)

No	Name of Ward	Number of traps laid	Number of traps with rats	Traps with rats for 100 traps laid	Number of rats caught	Number of rats caught for 100 traps laid
1	High School Road	135	"	1	"	28.9
2	Post Office Road	73		0.1		89.0
3	Bazaar area					104.3
4	Janappar area					"
5	Weaver				40	
6	"					

may be true or may be an accident due to the fewer number of traps laid in this area

Eight hundred and fifty-six traps were distributed in Hosur town in 127 of which rats were obtained, the number of rats being 302. The average works out at 35 rats in 15 traps for every 100 traps laid in the area. The non-appearance of plague in Hosur in the year 1927 has given the rats an opportunity to reach their normal density. Hirst (1927) considers the average of 30.2 rats for 100 traps obtained by the Indian Plague Commission for Madras as a fairly representative figure.

The villages of Pedda Jibi and Dinnur gave fairly high yields and this is not surprising in view of the housing conditions prevailing in the locality.

#### SPECIES AND SEX OF RATS

The rats trapped belong to the species *Rattus rattus*. Both varieties—the white bellied and the brown bellied—were represented, the latter predominating (*vide* Table IV). The white bellied variety formed nearly one-fifth of the total catch.

TABLE IV

No	Area	NUMBER OF RATS ( <i>R. rattus</i> )			NUMBER OF WHITE BELLED RATS			NUMBER OF BROWN BELLED RATS		
		Total	Males	Females	Total	Males	Females	Total	Males	Females
1	High School Road	39	11	28	7	2	5	32	9	23
2	Post Office Road	65	17	48	17	5	12	48	12	36
3	Bazaar area	72	20	52	8	1	7	64	19	45
4	Janappar area	25	12	13	2	1	1	23	11	12
5	Weaver area	40	12	28	10	2	8	30	10	20
6	Vanyar area	50	15	35	9	1	8	41	14	27
7	Hindu area	10	6	4	2	0	2	8	6	2
8	Taluk Office area	1		1	1		1	0		
	TOTAL	302	93	209	56	12	44	246	81	165
1	Therpet	37	14	23	10	4	6	27	10	17
2	Chanasundaram	7	2	5	2	0	2	5	2	3
3	Alasanattam	8	4	4	2	1	1	6	3	3
4	Pedda Jibi	11	2	9	1	0	1	10	2	8
5	Basti									
6	Dinnur	10	3	7				10	3	7

Regarding sexes there is a striking disproportion between males and females. Females preponderate, 69.2 per cent of the total number of rats obtained belonging to this sex. This disparity between the two sexes is seen in both the varieties of rats, the males in each case being only 21.4 and 23 per cent of the total respectively.

A few pregnant rats were encountered but in the absence of a systematic examination of all rats caught to see whether they were pregnant or not, it is not possible to say definitely if the rat population at Hosur is on the increase.

### BAIT

In the course of trapping operations, an attempt was made to evaluate the efficiency of different baits to attract rats. The following baits were tried (1) ground-nut, (2) cakes made of 'ragi,' (3) salt fish, (4) onions, (5) chillies, and (6) half fried cocoanut.

The baits used in the bazaar area and the Post Office Road where the density of rat population is very high have not been considered in assessing the efficiency of different baits. The owners of houses where traps were distributed very often added baits of their choice, in spite of instructions not to do so. This will vitiate the conclusions, but a general idea of the comparative values can be formed by a reference to Table V.

TABLE V

No	Baits used	Number of traps laid	Number of traps with rats	Number of rats caught	Number of rats per 100 traps
1	Ground-nuts	197	23	55	28
2	Ragi cake	157	26	43	27.4
3	Fried cocoanut	35	7	9	25.7
4	Salt fish	113	17	27	24
5	Onions	201	19	37	18.4
6	Chillies	25	2	2	8

Ground-nuts and cakes made of 'ragi,' the local grain come at the head of the list as regards efficiency in attracting rats. Next in order come the salt fish and the half fried cocoanut.

### INCIDENCE OF PLAGUE IN TRAPPED RATS

To form an estimate of the number of trapped rats found infected, nearly 200 rats collected from different areas were opened up and examined for any pathological signs of the disease. A smear from the spleen was examined for plague bacilli in every case. The results were entirely negative.



## FLEA SURVEY

*General*—Five species of fleas were found to occur on Hosur rats 5,417 rat-fleas were collected from Hosur and the adjoining villages, of which Hosur alone contributed 4,097 Except for two specimens of *Ctenocephalus canis* and one of *Leishnophaga gallinacea*, all the remaining fleas belonged to the genus *Xenopsylla* All the three Indian species of *Xenopsylla* were represented, *X. brasiliensis* alone forming nearly 63 per cent of the whole The highest number of fleas obtained on a single rat was 110 and there was only one rat and that a young one, without any fleas found on its body

*Pulex irritans*, the human flea, is so common in houses that it has almost become a pest in the locality The crevices and holes in the mud walls of houses afford them excellent shelter and large numbers of them can be taken without any difficulty 116 specimens thus caught were examined and all of them with the exception of three *Xenopsylla* proved to be *Pulex irritans* Although *Pulex* is rarely found on rats, it is surprising that out of 5,417 rat-fleas examined, not one belonged to this genus

*Sex proportions*—More than 62 per cent of the rat-fleas collected in Hosur town were males The percentage of males varied from 53 to 63 in the various villages examined The preponderance of the male sex is brought out very strikingly when the figures for the various divisions are examined

TABLE VI (a)

No	Area	Total fleas	MALE		FEMALE	
			Number	Percentage of total	Number	Percentage of total
1	High School Road	498	301	60.4	197	39.6
2	Post Office Road	716	441	61.6	275	38.4
3	Bazaar area	804	526	65.4	278	34.6
4	Janappar area	391	238	60.9	153	39.1
5	Weaver area	705	463	65.7	242	34.3
6	Vanyar area	868	501	57.7	367	42.3
7	Hindu area	112	74	66.1	38	33.9
8	Taluk Board area	3	1		2	
	TOTAL	4,097	2,545	62.1	1,552	37.9

TABLE VI (b)

No	Area	Total fleas	MALE		FEMALE	
			Number	Percentage of total	Number	Percentage of total
1	Therpet	759	407	53.6	352	46.4
2	Chanasundaram	138	74	53.6	64	46.4
3	Alasanattam	110	62	56.4	48	43.6
4	Pedda Jibi	208	132	63.5	76	36.5
5	Basti					
6	Dinnur	95	54	56.8	41	43.2
7	Wild places	10	7		3	

Table VII (a) gives the percentages of males and females in different areas for the three species of *Xenopsylla*. The males of *X. cheopis* and *X. brasiliensis* constitute 66.4 and 64.1 per cent of their total, while the distribution of sexes in *X. astia* is irregular. Nearly 56 per cent of *X. astia* were females.

The abnormal high proportion of males among *X. cheopis* and *X. brasiliensis* and of females among *X. astia* is also brought out when the figures for fleas collected in the adjoining villages are examined (*vide* Table VII (b)).

TABLE VII (b)

No	Name of area	<i>X. cheopis</i>			<i>X. brasiliensis</i>			<i>X. astia</i>		
		Total	Percentage Male	Percentage Female	Total	Percentage Male	Percentage Female	Total	Percentage Male	Percentage Female
1	Therpet	241	53.5	46.5	429	55.9	44.1	89	42.7	57.3
2	Chanasundaram	23	69.6	30.4	35	74.3	25.7	79	40.5	59.5
3	Alasanattam	44	68.2	31.8	33	66.7	33.3	33	30.3	69.7
4	Pedda Jibi	49	65.3	34.7	156	64.1	35.9	3		
5	Basti									
6	Dinnur	32	40.6	59.4	60	68.3	31.7	3		

TABLE VII (a)

No	Name of area	<i>X cheopis</i>			<i>X brasiliensis</i>			<i>X astia</i>			<i>Clunoccephalus</i>		
		Total	Per cent Male	Per cent Female	Total	Per cent Male	Per cent Female	Total	Per cent Male	Per cent Female	Total	Per cent Male	Per cent Female
1	High School Road	113	65.5	34.5	335	63.9	36.1	48	29.2	70.8	2		
2	Post Office Road	155	61.9	38.1	524	62.6	37.4	37	46.0	54.0			
3	Bazaar area	218	68.0	32.0	541	65.4	34.6	45	53.3	46.7			
4	Janappara area	84	72.6	27.4	205	63.0	37.0	102	47.1	52.9			
5	Weaver area	195	65.1	34.9	464	67.2	32.8	46	52.2	47.8			
6	Vanyara area	351	57.0	43.0	435	60.5	39.5	82	46.3	53.7			
7	Hindu area	36	75.0	25.0	63	66.7	33.3	13	38.5	61.5			
8	Taluk Board area	1			1			1					

## SPECIES DISTRIBUTION OF FLEAS

As between the three species of *Xenopsylla*, *X. brasiliensis* is the predominant flea of Hosur. Out of 4,097 fleas collected in Hosur town, as much as 2,568 (62·8 per cent) belonged to this species. Next in order comes *X. cheopis* which constitutes about 28 per cent of the total, *X. astia* being only a little more than 7 per cent (*vide* Table VIII).

The distribution of the three species in the villages surveyed, corresponds fairly well with the distribution in the town with the exception of two villages where *X. brasiliensis* occupies only a secondary place.

Except for the 'Janappar' and 'Vanyai' areas which show a higher percentage of *X. astia* and *X. cheopis* respectively, a marked uniformity of distribution as regards the proportions of the 3 species in the whole area is noticed.

Another interesting feature was the distribution of the different species of fleas on rats obtained from the fringes of the town, as compared with those obtained from the interior.

	<i>X. cheopis</i> index	<i>X. brasiliensis</i> index	<i>X. astia</i> index
Rats from periphery	4·8	7·7	2·4
Rats from interior	2·7	7·9	0·6

It is found that the *Astia* index for rats obtained from the outermost zone on the periphery of the town is four times higher than that for rats from the heart of the town away from the fields. It will be interesting to see whether observations in other places will confirm this.

TABLE VIII

No	Name of area	Total number of fleas	<i>X. cheopis</i>		<i>X. brasiliensis</i>		<i>X. astia</i>		<i>Ctenocephalus</i>	
			Total	Percentage of all fleas	Total	Percentage of all fleas	Total	Percentage of all fleas	Total	Percentage of all fleas
1	High School Road	498	113	22·7	335	67·3	48	9·6	2	0·4
2	Post Office Road	716	155	21·6	524	73·2	37	5·2		
3	Bazaar area	804	218	27·1	541	67·3	45	5·6		
4	Janappar area	391	84	21·5	205	52·4	102	26·1		
5	Weaver area	705	195	27·7	464	65·8	46	6·5		
6	Vanyai area	868	351	40·4	435	50·2	82	9·4		
7	Hindu area	112	36	32·1	63	56·3	13	11·6		
8	Taluk Board area	3	1		1		1			
TOTAL		4,097	1,153	28·1	2,568	62·8	374	9·1		
1	Therpet	759	241	31·7	429	56·5	89	11·8		
2	Chanasundaram	138	23	16·7	35	25·4	79	57·8		
3	Alasanattam	110	44	40·0	33	30·0	33	30·0		
4	Pedda Jibi	208	49	23·6	156	75·0	3	1·4		
5	Basti									
6	Dinnur	95	32	33·6	60	63·2	3	3·2		
TOTAL		5,407	1,542	28·5	3,281	60·8	581	10·7		

## FLEA INDEX

From the epidemiological point of view, the two basic data which are most important in relation to the potential incidence of plague in any locality are (1) the average number of fleas per rat, i.e., the general flea index, and (2) the average number of each species of flea per rat—the specific flea index

Table IX gives the general flea index for the different areas in Hosur and the adjoining villages

TABLE IX

No	Name of area	Number of rats trapped	Number of fleas captured	Number of fleas per rat, 'Flea index'	Number of <i>X. cheopis</i> per rat	Number of <i>X. brasiliensis</i> per rat	Number of <i>X. astia</i> per rat
1	High School Road	39	498	12.8	2.9	8.6	1.2
2	Post Office Road	65	716	11.0	2.4	8.1	0.6
3	Bazaar Road	72	804	11.2	3.0	7.5	0.6
4	Janappar area	25	391	15.6	3.4	8.2	4.1
5	Weaver area	40	705	17.6	4.9	11.6	1.2
6	Vanyar area	50	868	17.4	7.0	8.7	1.6
7	Hindu area	10	112	11.2	3.6	6.3	1.3
8	Taluk Board area	1	3				
TOTAL		302	4097	13.6	3.8	8.5	1.2

1	Therpet	37	759	20.5	6.5	11.6	2.4
2	Chanasundaram	7	138	19.7	3.3	5.0	11.3
3	Alasanattam	8	110	13.8	5.5	4.1	4.1
4	Pedda Jibi	11	208	18.9	4.5	14.2	0.3
5	Easti						
6	Dimnur	10	95	9.5	3.2	6.0	0.3
TOTAL		375	5407	14.4	4.1	8.7	1.5

The general flea index for Hosur town is 13.6, while the index for the different wards range between 11 and 17.6

The number of fleas per rat is higher for the villages as a whole and this may very likely be due to the fewer number of rats trapped in those places

Three rats—white bellied *R. rattus*—obtained from traps set in fields yielded only 10 fleas, all of them being '*X. brasiliensis*'. The significance of this was not immediately realized, but in future more attempts will be made to catch rats in fields as well as in houses to see what light this throws on the flea population, for the presence of *X. brasiliensis* alone on field rats, while the 'town' rats had *X. cheopis* as well, is very suggestive

#### SPECIFIC FLEA INDEX

The number of each species of *Xenopsylla* per rat is also given in Table IX. The *cheopis* index for the whole of Hosur town is 3.8 while the index for *X. brasiliensis* and *X. astia* comes to 8.5 and 1.2 respectively.

It is significant that the Vanyar area which is free from plague at present and which recorded about 37 per cent of the total attacks during the last plague season (1926) should show the highest *cheopis* index while the Weaver area where 57 per cent of the cases have occurred during September this year should record the abnormally high index of 11.6 for *X. brasiliensis*. This coupled with the fact that a large number of *X. brasiliensis* per rat is found in the area, leads to the suspicion that *Xenopsylla brasiliensis* may be an equally efficient vector as *X. cheopis* in the transmission of the disease under identical climatic conditions. Though the magnitude of the specific flea index in any given locality is no criterion of the efficiency of that particular species to act as a vector, it is possible to surmise that if the index for that flea is very high in a plague area, the species is likely to be incriminated in the transmission of the disease. The presence of 33 fleas of which all except one were *X. brasiliensis* on a rat which was seen to fall and die and which was found to have died of typical plague when examined immediately afterwards, adds indirect force to the argument that *X. brasiliensis* may possibly prove to be an efficient vector in nature. This species has not received much attention and very little is known regarding its powers of experimental plague transmission and its bionomics.

Of the villages, Chanasundaram which remained free from plague for the last seven years yielded 138 fleas distributed on 7 rats, the percentage of *X. astia* alone being as high as 58. The number of *X. astia* obtained per rat in the village was 11.3, the highest figure recorded for this species during this survey.

#### DISTRIBUTION OF FLEAS ON RATS

Except one young rat which did not harbour any fleas, every rat had fleas on them. Nearly 69 per cent of the rats with fleas contained all the three species of *Xenopsylla*. The number of rats with both *X. cheopis* and *X. brasiliensis* on them is 87, i.e., 29 per cent of the rats with fleas. Table X gives the details regarding distribution of fleas.

TABLE X

No	Name of area	Rats with fleas	Rats with all 3 species of <i>Xenopsylla</i>		Rats with <i>X. cheopis</i> and <i>X. brasiliensis</i>		Rats with <i>X. cheopis</i> and <i>X. asia</i>		Rats with <i>X. cheopis</i> alone		Rats with <i>X. asia</i> alone	
			Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
1	High School Road	38	31	81.6	5	13.2	0		2	5.2	0	
2	Post Office Road	65	40	61.5	23	35.4	2	3.1	0		0	
3	Bazaar area	72	47	65.3	25	34.7	0		0		0	
4	Janappar area	25	19	76.0	6	24.0	0		0		0	
5	Weaver area	40	25	62.5	14	35.0	1	2.5	0		0	
6	Vanyar area	50	36	72.0	12	24.0	1	2.0	0		1	2.0
7	Hindu area	10	8	80.0	2	20.0	0		0		0	
8	Taluk Office area	1	1		0		0		0		0	
TOTAL		301	207	68.8	87	28.9	4	1.3	2	0.7	1	0.3

Another important feature was the presence of *X. cheopis* in 300 out of 301 rats with fleas, showing that almost every rat in Hosur town was *cheopis* infested, which rather weakens the suspicion that *X. brasiliensis* also carries plague

## SUMMARY

## RATS

1 The density of rat population as judged by the number of rats trapped for every 100 traps laid, i.e., 35, is fairly high for the whole area, anything over thirty being abnormal. The bazaar and the commercial portion of the town as usual yields the highest figures.

2 Two varieties of *Rattus rattus*—the white bellied and the brown bellied varieties—are present, nearly 75 per cent belonging to the latter kind.

3 There is a great disproportion in the distribution of the two sexes, nearly 70 per cent of the rats being females. This unequal distribution of the two sexes is seen in both the varieties of *R. rattus*.

4 Different baits were tried. Ground-nuts and cakes made of 'ragi,' the grain grown locally, proved to be most efficient. Other baits which were also found useful in attracting rats were the half fried cocoanut and dried salt fish.

5 200 trapped rats were examined for signs of plague with negative results.

## FLEAS

1 5,417 rat fleas were collected on rats All these, excepting three belonged to the genus *Xenopsylla* The three Indian species of *Xenopsylla* are represented

2 *Pulex irritans* was very common in the houses but was not found to occur on rats

3 More than 60 per cent of the fleas collected belonged to the male sex *X cheopis* and *X brasiliensis* consistently showed a very high proportion of males in all the areas while the distribution of the sexes in *X astia* was found to be irregular, the females being slightly in excess on the whole

4 As between the 3 species of *Xenopsylla*, *X brasiliensis* is the predominant flea of Hosur (62.8 per cent) Next in order comes *X cheopis* constituting about 28 per cent of the total Nearly 69 per cent of the rats had all the three species of *Xenopsylla* on them The distribution of the species in the villages agrees fairly well with that of the town

5 Almost every rat in Hosur was *cheopis* infested Despite this, the possibility of *X brasiliensis* being a vector should still be considered

6 There is some evidence that the distribution of flea species differs in the town proper and in its outskirts

7 The number of fleas per rat—the general flea index—for Hosur town is 13.6 The variations in the index for different areas range between 11.0 and 17.6

8 The number of each species of flea per rat is as follows —

	For Hosur Town	For Hosur Town and Environs
1 <i>X cheopis</i>	3.8	4.1
2 <i>X brasiliensis</i>	8.5	8.7
3 <i>X astia</i>	1.2	1.5

## REPORT NO II

## A RAT-FLEA SURVEY OF THE WESTERN HALF OF THE BELLARY DISTRICT AND GUNTAKAL OF THE ANANTAPUR DISTRICT

BY

N NATARAJAN, M B, B S, B S S C

THIS area is situated on the Deccan tableland All the places surveyed are about 1,500 feet or more above sea-level

The time of survey was from the 17th October, 1928 to 20th January, 1929,—the usual plague season The following table gives the figures for the items noted under each column

Hospet is usually cooler during this period than the above figures indicate so this year was unusually warm

The places surveyed have a scanty annual rainfall, the average being about 20 inches The usual rainy months are from June to September and October



TABLE I

Name of station	Time of survey	Dry bulb Mean daily temperature 8—9 a.m.	Wet bulb Mean daily temperature 8—9 a.m.	Relative humidity	Mean satu- ration defi- ciency in inches 8—9 a.m.
1 Bellary Town and Cantonment	Oct 17—Nov 16	76.6°F	69.2°F	66.8	0.300
2 Hospet one Union and two villages	Nov 16—Dec 10	76.8°F	69.5°F	70.8	0.303
3 Harpanahalli and six villages	Dec 10—Dec 27	70.2°F	62.6°F	68.0	0.201
4 Kotturu	Dec 27—Jan 4	73.4°F	65.5°F	69.2	0.300
5 Guntakal	Jan 5—Jan 20	74.0°F	66.7°F	69.6	0.277

*Bellary* is a municipality with a population of thirty-seven thousand. It consists of the following three distinct areas—(1) the main portion called Brucepettah which is the centre of commercial traffic, godowns and bazaars, (2) the extensions consisting of pucca houses and bungalows separated from one another by open spaces and compound walls and more sanitarily kept, (3) Cowl Bazaar, another suburb, at a distance of about one mile, containing nearly one-third the population of the municipality and inhabited by the middle and lower classes of people. In this suburb there are no large bazaars or godowns.

*Bellary Cantonment and Fort* is a distinct entity under the control of the military authorities. This station has about two hundred pucca buildings built over a large area containing the civil and military population. The Fort nearer the town contains a few shops and bungalows. Though surrounded on two sides by Bellary Town, the rat-flea population is strikingly different in respect of the species distribution, as is seen in the tables under the fleas section.

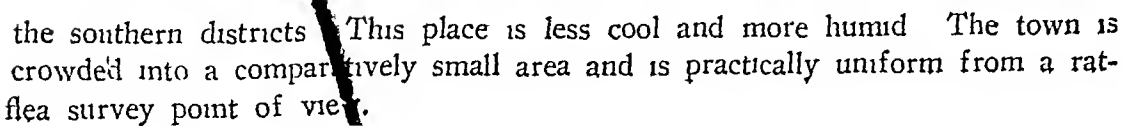
*Hospet* is a municipality with a population of eighteen thousand. The climate is cooler and more humid than that of Bellary. There are no great differences between different portions as regards sanitation, overcrowding, etc. There is a suburb of the town one mile off which is somewhat cleaner and which was once the main part of Hospet having been deserted due to plague years ago. Hospet is the most insanitary of the places surveyed.

During my stay at Hospet three more places were surveyed of which one 'Union' of 3,500 inhabitants, namely, Narayanadevarakeri, nine miles off from Hospet, was passing through an outbreak of plague and most of it was evacuated.

*Harpanahalli*—2,000 feet above the sea-level—is the coolest of the surveyed places. It is about thirteen miles from the Mysore borders. It is a major union of about 8,000 inhabitants. This and the surrounding parts appear to be an endemic centre of plague. Plague has occurred almost every year.

A MAP OF THE BELLARY DISTRICT

Scale 1 inch = 27½ miles.



Guntakal belongs to the Anantapur district. It has a population of 12,000. It is also 1,500 feet above sea-level. Its climate is a little more humid than that at Bellary and is hotter than any of the stations surveyed in the Bellary District. Guntakal is a railway junction where five railway lines meet, the union consists of three distinct suburbs separated by one mile or so from one another. The railway quarters around the junction consist of about 200 to 300 pucca buildings inhabited by the railway officers and subordinates. These buildings are separated by large open spaces from one another. The porters' and coolies' lines belonging to the railway are different. They are near the goods shed and because of the nature and habits of the people therein, the rat-flea population is different in species distribution to that which obtains in the railway quarters. This is noted in Table VIII. The second suburb of Guntakal is Timmanacherla about a mile from the railway station. It contains the main bazaar and the weekly shandy or market is held here. Even within this small suburb there is a difference in the flea distribution between the bazaar area and the domestic quarters. This is brought out in Table VIII. The third suburb is old Guntakal about one mile from Timmanacherla and more distant from the railway station. This is inhabited by ryots and agriculturists.

*Conditions with regard to plague*—There is no difference in the habits of the people of these places. The usual habit is for the people to sleep on the floor.

With regard to the housing conditions a word may be said in respect of their roofing. Above wooden rafters or bamboo sticks are placed bamboo thatties over which are laid twigs and leaves which layer is covered by mud and earth. The whole roofing constitutes a thickness of nine to twelve inches. Such a roofing affords excellent nesting conditions for rats. With such a type if the walls also are of mud or of burnt stones laid in mud leaving large holes, which often is the case, an ideal home for the rats is afforded and burrows under the ground can easily communicate through the wall with the ceiling above. This kind of roofing is a feature of all houses except the bungalows and other high class cement and stone structures.

The majority of the buildings because of this kind of roofing are dark in their interior. Consequently the houses are ill-lighted and ill-ventilated. The houses are so closely built that there is continuity for rats to move freely from one place to another.

There is no system of drainage for any of the places. Disposal of rubbish is especially inefficient at Hospet.

## EXPORT

*Movement of grains*—The chief common export of all the places surveyed is ground-nuts and next cotton and cotton seeds. Hospet exports jaggery in addition. Gingelly seeds from Kotturu are a special export to Vellore and some places in the Tanjore district. The ground-nuts are exported to Marmagoa mostly and to Madras from Guntakal. Gingelly and cotton seeds and cholam grains from Kotturu may be a source of infestation with rat-fleas. The outbreaks of plague

at Vellore may have some connection with the import of gingelly seeds from Kotturu into this town

### IMPORT

Import of commodities from the Bombay Presidency, the Nizam's territory and from the Mysore Province is attended with the risk that these will be infested with *X cheopis* or its developmental forms. *Wheat* comes from Gadag and Bijapur of the Bombay Presidency, Gadag being an endemic plague centre. The Nizam's State export cholam and black gram, while dhal comes from Agra and the Punjab. Harpanahalli imports its grains from Davanagere of the Mysore State, another endemic focus.

Rice is imported from the districts of Kistna and Godavari where there has been no plague.

### RATS

The rodents trapped were 2,556, of which four were bandicoots, four were mice and the rest were rats. All rats belonged to the species *Rattus rattus*. The colour of the bellies was grey and brown and the white-bellied rats were at their maximum at Harpanahalli. The number of white-bellied rats was 37, 20, 146, 39 and 2 at Bellary, Hospet and villages, Harpanahalli and villages, Kotturu and Guntakal respectively.

The next table shows the density of rat population as judged by the number of rats per 100 traps laid and also the percentage of female rats which is always greater than that of males for each place.

TABLE II

Name of place	Number of traps laid	Total rats trapped	Number of rats per 100 traps laid—density	Percentage of female rats
1 Harpanahalli	500	442	88	74.0
2 Six villages surrounding Harpanahalli	277	182	66	74.2
3 Kotturu	350	257	73	65.4
4 Hospet	721	342	47	70.8
5 A Union and two villages surrounding Hospet	370	134	36	80
6 Bellary	1,487	759	51	62.3
7 Guntakal	738	330	45	63.3
8 Bellary Cantonment	200	110	55	79.1
TOTAL	4,743	2,556	55	68.4

At Bellary 110 rats were examined for plague infection macroscopically and by spleen smears. All were negative. All the 176 rats from Hospet and the surrounding villages were similarly examined and found negative.

Two mice at Bellary harboured one *X. astia*, four *X. cheopis* and one louse. Two mice trapped at Guntakal from domestic quarters harboured no fleas.

Four bandicoots were trapped in the whole survey. They harboured more fleas than the rats. One can expect on them three to four times the number of fleas found on a rat considering that a bandicoot is three or four times as large in its surface area as a rat. My number of bandicoots was too small to draw conclusions.

TABLE III

Field rats are those trapped in the fields far away from human habitation. They do not harbour *X. cheopis* but only *X. astia* or no fleas.

Name of place	Field rats	<i>X. astia</i>	<i>X. cheopis</i>	Other ecto-parasites
1 Bellary	5	9		11 ticks
2 Hospet	2	1		2 "
3 Harpanahalli	2			
4 Narayanadevarakeri	2 bandicoots in one trap	22	16	4
	12 rats and 1 bandicoot in a trap	45	229	
5 Guntakal	One rat and a bandicoot in a trap	39		24

## FLEAS

The prevailing rat-fleas in the surveyed areas are *Xenopsylla astia* and *Xenopsylla cheopis* and at Harpanahalli alone in a particular portion of the town there is *Xenopsylla brasiliensis* in addition.

The maximum number of fleas on a rat at Bellary was 35, of which 30 were *X. cheopis* and 5 were *X. astia*. This rat was trapped in a wheat shop. At Hospet 47 was the maximum on a rat, of which 25 were *X. cheopis*. At other places the maximum was between 16 and 23.

TABLE IV  
Specific and total flea indices, etc

Name of place	Rats examined	Total flea index	<i>astia</i> index	<i>brasiliensis</i> index	<i>cheopsis</i> index	Percentage of rats without <i>cheopsis</i>
1 Harpanahalli	442	61	0.02	0.04	6.0	0.0
2 Six villages surrounding Harpanahalli	182	6.0	0.6		5.4	0.0
3 Kotturu	257	64	0.9		5.5	0.0
4 Hospet	342	5.9	1.3		4.6	1.4
5 A Union and two villages near Hospet	134	7.8	1.8		6.0	15.3
6 Bellary Town	759	5.9	2.5		3.4	20.0
7 Bellary without extensions and Cowl Bazaar suburb	575	6.0	2.0		4.0	1.5
8 Guntakal	330	4.2	2.8		1.4	60.0
9 Bellary Cantonment	110	7.3	7.2		0.1	92.7
* The Union alone	66	11.4	2.3		9.1	0.0

\* This Union is Narayanadevarakeri. At the time of survey it was passing through an outbreak of plague, and was evacuated except for the bazaar and the Brahmin Street. The rats trapped in this Union show the highest *cheopsis* index and also total flea index. This may be partly due to concentration of the fleas on the surviving rats.

Numbers 6 and 7 in the foregoing table illustrate that closeness of houses conduces to distribution of *X cheopsis* on almost all the rats.

The next table shows very well the association of the *cheopsis* index with deaths from plague.

TABLE V

	Harpanahalli	Kotturu	Hospet	Bellary	Guntakal
Deaths from plague per mille of population for 1914-28 per year	10.5	8.1	5.3	3.6	1.4
Present <i>cheopsis</i> index of each place	6.0	5.5	4.6	3.4	1.4

The next table shows the association between climate and the species of rat-flea prevalent

TABLE VI  
*Percentage distribution of the three species of rat-fleas*

Name of place	Total fleas	Percentage <i>astia</i>	Percentage <i>cheopis</i>	Percentage <i>brasiliensis</i>
1 Harpanahalli	2 857	0.3	93	67
2 Six villages near Harpanahalli	1,095	11	89	
3 Kotturu	1,639	14	86	
4 Hospet	2 049	24	76	
5 A Union and two villages near Hospet	1 041	23	67	
6 Bellary Town	4 482	43	57	
7 Guntakal	1 365	67	33	
8 Bellary Cantonment	805	99	1	

The climate is the coolest at Harpanahalli and is less and less cool in the places that follow. It is to be noted that this order is the ascending order for

TABLE VII  
*Sex distribution of the three species*

Name of place	<i>X astia</i>		<i>X brasiliensis</i>		<i>X cheopis</i>	
	Male	Female	Male	Female	Male	Female
1 Harpanahalli	40.0	60.0	40.5	59.5	53.7	46.3
2 Six villages near Harpanahalli	35.3	64.7			50.7	49.3
3 Kotturu	33.3	66.7			59.6	40.4
4 Hospet	34.9	65.1			59.6	40.4
5 A Union and two villages near Hospet	37.7	62.3			58.6	41.4
6 Bellary Town	38.6	61.4			60.3	39.7
7 Guntakal	40.0	60.0			63.1	36.9
8 Bellary Cantonment	38.9	61.1				

*X astia* but the descending order for *X cheopis* Bellary Cantonment is one step out of place of its climatic order. Although geographically one with Bellary town it is essentially different in all other respects affecting fleas.

In the foregoing table it is noted that *X astia* has a larger female population and that *X cheopis* has a larger male population. The percentage for *X astia* appears to be fairly uniform, while the percentage of female *X cheopis* varies rather more, the maximum percentage of female *X cheopis* being at Harpanahalli and its surrounding villages—places of highest incidence of plague, the lowest percentage of female *X cheopis* being at Guntakal, a place of smallest incidence of plague among the surveyed stations.

The next table shows differences in rat and rat-flea density according to differences in the several portions of the surveyed stations as regards grain movements, sanitation, and nature of area.

TABLE VIII

Name of station and area	Rats trapped	Rats per 100 traps laid = density	<i>astia</i> index	<i>cheopis</i> index	Description of the areas
<i>Bellary</i> —					
Only gunny bag shops in the Bangalore Road	37	23	26	72	
Godowns, grain shops, etc	187	150	18	47	
Brucepettah	493	67	19	40	Containing nearly three-fourths of the population of Bellary, godowns, bazaars, various shops and quarters for low class people
Cowl Bazaar	62	28	61	29	A suburb 1 mile from the above inhabited by ryots and labourers. No godowns or grain centres.
Extensions	91	30	39	08	Large compounds with bungalows. Gandhi Nagar and Satyanarayanapeth.
Cantonment and Port	110	55	72	01	Near the town very sanitary and controlled by the military.
<i>Guntakal</i> —					
Railway quarters	25	24	14	13	Pucca buildings placed at a distance from one another but in frequent communication with the town and junction.
Porters and coolies lines	32	52	18	45	Four rows of small single room tenements contiguous with one another built in barrack-like fashion near railway goods shed.



TABLE VIII—*concl'd*

Name of station and area	Rats trapped	Rats per 100 traps trapped = density	<i>astia</i> index	<i>cheopis</i> index	Description of the areas
Timmanacherla domestic quarters	34	27	3.2	0.3	Clean quarters
Timmanacherla Bazaar area	32	36	2.3	4.4	Clean but the centre for traffic in grains, etc.
Old Guntakal	96	48	4.6	0.8	One mile away from Bazaar areas and not of any grain importance
Bellary — Bazaar area	279	153	2.1	4.2	
Extension and domestic quarters	184	26	4.3	1.5	
Hospet — Bazaar area	73	72	1.4	5.1	
Domestic quarters	269	44	1.4	4.4	
Harpanahalli — Bazaar area	99	103	0.0	4.8	<i>Brasiliensis</i> index 1.5
Domestic quarters	343	85	0.03	6.3	0.1
Kotturu — Bazaar area	61	86	1.9	5.4	
Domestic quarters	196	70	0.6	4.0	
Guntakal — Bazaar area	32	36	2.3	4.4	
Domestic quarters	298	46	2.8	1.0	
Total — Bazaar area			1.5	4.8	
Domestic quarters			1.8	3.4	

In the foregoing table it is noted that the bazaar areas of a station have a denser rat population than the domestic quarters. Also the infestation with *X cheopis* is more in the bazaar areas than in the domestic quarters except at Harpanahalli where the domestic quarters are more infested. Perhaps this is due to greater facilities existing in these quarters for flea-breeding and multiplication.

*Other ectoparasites* — Most of them were ticks and mites. About 60 lice and a dozen beetles were also seen.

For convenience of information the next table summarizes the data on which the previous tables are based

TABLE IX

Showing total figures for rats, fleas and other ectoparasites

Name of place	Population	Rats trapped	<i>X astia</i>	<i>X brasiliensis</i>	<i>X cheopis</i>	Total fleas	Other ectoparasites
1 Bellary Town	37,567	759	1,925		2,557	4,482	509
2 Bellary Cantonment and Fort		110	795		10	805	23
3 Hospet	18,337	342	490		1,559	2,049	323
4 A Union and two villages near Hospet	5,905	134	236		805	1,041	99
5 Harpanahalli	7,464	442	10	195	2,652	2,857	127
Six villages near Harpanahalli	5,607	182	116		979	1,095	92
otturu	6,272	257	225		1,414	1,639	307
Guntakal	12,519	330	920		445	1,365	545
TOTAL		2,556	4,717	195	10,421	15,033	2,025

## DISCUSSION

Bellary Cantonment has practically a *cheopis*-free rat-flea population. Rats trapped in the fields had only *X astia* and it looks as if *X cheopis* was not indigenous in this district. At Harpanahalli the rat-flea population was almost *astia* free. The disappearance of *X astia* from this last station may be explained this way. Owing to a series of epizootics spread over successive years this species being gradually reduced in numbers is dying out while *X cheopis* has survived these outbreaks and multiplied so much that the present flea population is most *X cheopis*.

The point to be considered is how far the *cheopis* infestation of the rats of these areas is of any consequence to the places to which there is export of some commodity or other from any of these areas. Harpanahalli with only *cheopis* is not an exporting centre of any importance. On the other hand its surrounding parts import all the necessaries from Davanagere of the P another place with frequent visitations of plague.

The presence of *X brasiliensis* at Harpanahalli and the 10  
flea in only two streets and not in any other streets was a fe. it  
if this flea was not an ordinary indigenous and either

imported or, if often imported, that there are special conditions which are unfavourable for its wider spreading

The movement of rats becomes more restricted if the houses are built at great distances from one another as in the extensions of Bellary and in the Bellary Cantonment. Closeness of houses with continuity of their roofings that prevails in all the areas surveyed conduces to more intermingling of rats from different areas. At the Bellary Cantonment the only bungalow the rats of which harboured *X. cheopis* is far removed by large open spaces as well as by a surrounding compound wall from the neighbouring bungalows. If this were otherwise, the rats of this bungalow may communicate with rats of the adjacent building and to that extent scatter the distribution of this flea.

Insanitation appears to affect differently the two species of rat flea *X. astia* and *X. cheopis*. *X. astia* seems to multiply in spite of good sanitary conditions. At the Bellary Cantonment the *astia* index was higher than anywhere else in the Bellary District and this place is the cleanest and most sanitary of the places surveyed. *X. cheopis* breeds and multiplies more under insanitary conditions and cleanliness of a place lowers the *cheopis* index as is seen in Table VIII.

A glance at the map of the Bellary district shows how it is wedged in between the Nizam's Dominions, the Bombay Presidency and the Mysore State. Originally the infestation with *X. cheopis* and infection with plague must have occurred through these areas. At present the rat-flea population is such that there is no risk of this district being any more infested with *X. cheopis* but the question of introducing infection from these areas is of importance. Even without importation of infection from outside the district, the plague infection may be kept up in the western half of the Bellary district by its being imported from one place to another as there is almost always plague in one spot or other except in the months of May and June when, during this short period, the epizootic may be mild or latent.

#### CONCLUSIONS

(1) The chief rat-fleas in these areas are *X. astia* and *X. cheopis*, the latter preponderating in cooler and in more insanitary areas. (2) *X. brasiliensis* was found only on rats from two adjoining streets of Harpanahalli which suggests recent importation.

My thanks are due to Lieut-Col H H King, I M S, Director, King Institute, Guindy, for his kind supervision and suggestions during the survey and for help in writing this report.

#### REPORT NO III

REPORT ON A RAT-FLEA SURVEY OF PERIAKULAM, KAMBAM VALLEY, AND DINDIGUL

BY

P V GEORGE, B A, L M & S

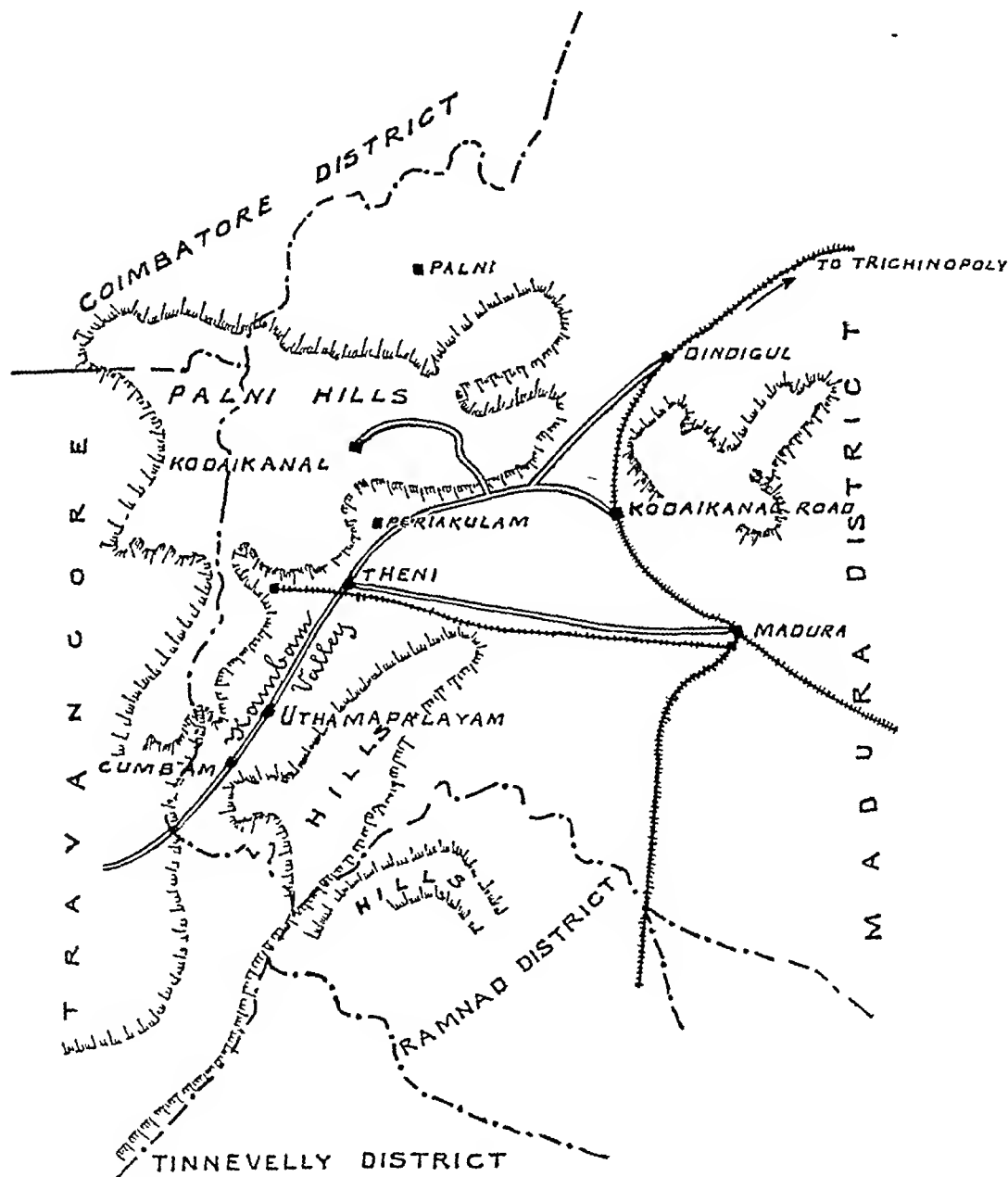
THE survey was done in the usual plague season of the district, commencing from the third week in October 1928 to the third week in January 1929. Four

centres, Periakulam, Theni, Uthamapalayam and Dindigul were chosen. From each centre a few villages were also selected, in all two municipal towns, two union towns and twenty villages were surveyed.

Two of the centres, *Theni* and *Uthamapalayam* form part of Kambam Valley in Periakulam taluq, situated at the foot of the Palni and Travancore hills. This

### A MAP OF THE KAMBAM VALLEY

Scale 1 inch = 23 miles



valley, from the perennial streams falling from its numerous forests and the cool wind which passes down it from the great hills in the west, is the greenest and pleasantest part of the whole district.

*Periakulam* is a municipal town, divided into two parts by the Varahanadi,—*Thenkaran* and *Vadakarai*. It is in close proximity with the Kodaikanal hills, the Palani hills, and the Travancore hills and is situated at an altitude of 954 feet.

*Dindigul* is situated between the Palni hills, and the Sirumalai hills, at an altitude of 924 feet above sea-level. It resembles Coimbatore district, in soil and climate. Next to Madura, it is the biggest municipal town in the district, with an area of 5.04 square miles. It is a very neat and sanitary town.

*Grains and grain movements*—The chief crops are rice, cholam, ragi, kambu, samai, varagu, horse gram, chillies, onions, cotton, gingelly, ground-nuts, sugarcane and tobacco. The chief place of production of these crops is Kambam Valley, and the chief places of disposal are *Theni*, *Dindigul*, *Madura* and *Virudhunagar*. Paddy is imported into Madura district from the Circars, Tanjore, Trichinopoly and Karachi because the local production is not sufficient for the whole district.

One chief feature of the Kambam Valley is the large number of weekly markets or 'shandies' mainly dealing in grains. The biggest of these 'shandies' is at *Theni*, where every Sunday on an average 1,000 cart loads of all sorts of grains are brought from all over Kambam Valley and disposed of, mainly through *Periakulam* to *Kodaikanal*, *Dindigul*, *Madura* and other places.

There are four toll gates around *Uthamapalayam* controlled by the District Health staff to check the movement of grains which have not been previously subjected to sun-disinfection—this is done under district orders only when human plague prevails.

*Climate*—The annual rainfall, average for 40 years, is 29.96 inches for *Dindigul*, 30.24 inches for *Periakulam* and 27.23 inches for *Uthamapalayam*. These places get rain during both monsoons, mostly from the North-East Monsoon.

Temperature and humidity observations made during the period of survey are given in table below, for each of the centres—

TABLE I

Name of place	Mean dry bulb temperature Fahr	Mean wet bulb temperature Fahr	Relative humidity	Mean saturation deficiency in inches
<i>Uthamapalayam</i> (Dec—Jan 1929)	75.83°	70.14°	74	0.26
<i>Theni</i> (Nov—Dec 1928)	79.73°	73.35°	72	0.28
<i>Periakulam</i> (Oct—Nov 1928)	80.6°	74.4°	72	0.28
<i>Dindigul</i> (Jan 1929)	79.32°	70.32°	64	0.38

*Dindigul* is the driest of all the places surveyed and *Uthamapalayam* is the coolest and the most humid.

*Plague*—The history of plague in Madura District hardly comprises two decades. It started first at Palni, infection having been introduced from Coimbatore district, and from there along the main routes to the interior, and finally established itself in the Kambam Valley, devastating many villages every year.

Periakulam had plague first in 1921 and had it for 5 years and disappeared since then. Theni had plague first in 1918, imported from Palni and Uthamapalayam in 1920, and ever since then the infection spread throughout Kambam Valley. There are, however, a few villages in Kambam Valley, which have escaped infection so far, in spite of the widespread epidemics all around, and two of them on survey showed a very low *cheopsis* index.

	Rats examined	Fleas collected	<i>astia</i> index	<i>cheopsis</i> index
(1) Kunnur	21	129	5.05	0.57
(2) Karuvel Naicken-patti	6	20	2.83	0.50

Dindigul had plague first about 1912, imported from Palni, and the worst year was 1920 and since then it has not reappeared in this town.

In all these places, the past plague figures show that the severest epidemics were in the years with heaviest rainfall. It was gathered from Dindigul and Kambam Valley that there used to be monkey-falls and a few crow-falls in addition to rat-falls during certain of their virulent epidemics. The monkeys were said to have had buboes as in human cases. In Uthamapalayam this year there is said to have been a few squirrel-falls during the human epidemic. During the time of survey, Uthamapalayam and 12 surrounding villages were passing through a severe epidemic of plague.

*Conditions affecting plague. Housing*—In Dindigul and Periakulam most of the houses have brick or stone walls and tiled roofing. In Theni and Uthamapalayam (Kambam Valley) most of the houses have only mud walls plastered with cow-dung and roofs of thatch. One special feature seen in Uthamapalayam and villages around it is a good number of houses provided with galvanized iron roofing. Although these are intended to be rat-proof, they do not function as such, because they have a wooden lining under the roof. Rats find very comfortable nesting places in the space between the roof and the wooden lining. The type of grain stores for these houses is a barrel-shaped receptacle plastered with cow-dung and designed to hold enough grain for one year's consumption.

*Sanitation*—Is quite unsatisfactory at Theni and Uthamapalayam, and the condition is still worse in the villages in Kambam Valley.

*Rats*—968 rodents were collected and examined. On the whole the rat population was very sparse, judged by the number of rats for 100 traps set. This is probably due to the organized rat catching done daily in each of these places for the last few years. This is borne out by the fact that the villages where no rat

catching is done showed a much denser rat population, the catch being as high as 50 rats for 100 traps set

TABLE II  
Rat density etc in the different places

Name of places	Number of traps laid	Number of rats caught	Number of rats for 100 traps	Percentage of female rats	Percentage of pregnant females to total females
Periakulam	2,485	317	12.75	72.24	27.00
Theni	1,458	202	13.85	62.38	28.60
Uthamapalayam	1,769	233	13.17	64.38	22.67
Dindigul	1,209	168	13.89	60.12	18.00

Special attempts were made to get as many field rats as possible, but the net result was not very encouraging. For 599 traps only 13 field rats were caught, but it was found they were a quite distinct variety (as yet unidentified) and different from the *Rattus rattus* got from adjoining houses. All of them had perfectly white bellies, and all the females showed 3 pairs of pectoral mammae and 3 pairs of inguinal mammae. They had proportionately longer tails than *R. rattus* the tail length being about 140 per cent of the head and body. Further they showed on an average 8 fetuses in utero, as contrasted with 5 for *R. rattus*.

In all species of rodents the females were over 60 per cent of the total and this was found true in all places. The percentage of pregnant female rats was highest at Theni (28.6) and lowest at Dindigul (18). This is probably due to the more favourable conditions for rat life prevailing in the former place, as a result of the obvious insanitary conditions, aggravated by the presence of the big market mainly dealing in grains, in contrast with the highly sanitary conditions obtaining in the latter place.

It was also observed that out of 968 rodents autopsied, 25 per cent showed the cystic stage of a tape-worm in the liver and certain livers showed as many as 12 cysts.

Spleen smears of all the specimens were stained and examined, without any positive result for plague. It is also worth while noting that out of 238 rats examined from heavily infected area, none showed any scars, perisplenitis or other evidences of healed plague infection.

All varieties of muridae, except *Rattus norvegicus*, were met with during the period of survey. Their numbers and respective flea indices are given in the tables below.

## Sex distribution of the rodents examined

	<i>R. rattus</i>	Field rats	Bandicoots	House mice	Musk rats	Total
Male	314	3	3	8	1	968
Female	603	10	17	8	1	

*Rat-fleas*—The species of fleas found in the survey were —

*Xenopsylla astia*,  
*Xenopsylla cheopis*,  
*Echidnophaga gallinacea*  
 and  
*Ctenocephalus canis*

The sex distribution, general and specific flea indices for each species of rodents, are all detailed in Tables III and V

The maximum number of fleas found on any one rat was 70 and the maximum number of *Xenopsylla cheopis* found on any one rat was 29. The other ectoparasites met with on rats during the survey were ticks, mites, beetles, bed-bugs, lice (including *P. capitis* of man), and pseudo-scorpions.

It was found that house mice, bandicoots, and musk rats also harboured *Xenopsylla cheopis* and that one mouse gave as many as 26 fleas of which 13 were *X. cheopis*.

*Flea indices*—The total and specific flea indices of *R. rattus* for the different places are given in Table III —

TABLE III

Places	Rats examined	Total flea index	<i>X. astia</i> index for <i>R. rattus</i>	<i>X. cheopis</i> index for <i>R. rattus</i>	<i>Echidnophaga gallinacea</i>	<i>Ctenocephalus canis</i>	Percentage of rats without <i>X. cheopis</i>
Uthamapalayam	233	8.18	2.30	4.69	277	0	3.36
Theni	202	6.95	4.87	2.00	15	0	42.00
Periakulam	317	5.07	3.82	1.02	72	0	50.00
Dindigul	168	7.26	4.87	2.39	0	1	26.40



The bazaar areas showed a rise in specific *chicopsis* index for *R. rattus*, in almost all places, except Uthamapalayam where the highest indices were found in the plague infected streets, in residential areas (See Table IV)

TABLE IV

A consolidated statement of rat density and flea indices according to different types of areas surveyed

Nature of areas	Number of rats got	Number of rats per 100 trap (rat density)	Total flea index for <i>R. rattus</i>	<i>Y. asia</i> index for <i>R. rattus</i>	<i>Y. chicopsis</i> index for <i>R. rattus</i>	REMARKS
<i>Bazaar areas—</i> Periakulam	62	14.00	4.27	3.15	1.12	1 <i>Echidnophaga</i> <i>gallinacea</i>
Theni	127	23.87	8.10	5.51	2.57	3 do
Uthamapalayam	30	12.24	7.00	2.87	4.13	
Dindigul	73	17.71	7.05	3.48	3.57	
<i>Better class residential</i> <i>areas—</i> Periakulam	105	11.11	4.54	3.51	1.03	
Theni	48	9.24	4.72	3.38	1.33	1 do
Uthamapalayam	153	14.05	7.20	2.66	4.54	52 do
Dindigul	20	11.83	6.05	4.95	1.10	
<i>'Paracheri' or low caste</i> <i>quarters—</i> Periakulam	80	16.42	4.54	4.00	0.54	.
Dindigul	66	20.56	7.82	6.17	1.64	1 <i>Ctenocephalus</i> <i>canis</i>
<i>Villages around each</i> <i>centre—</i> Periakulam	90	27.86	5.18	3.67	1.14	71 <i>Echidnophaga</i> <i>gallinacea</i>
Theni	27	26.83	5.52	4.55	0.55	11 do
Uthamapalayam	55	12.65	10.82	1.74	5.00	225 do
Dindigul	19	6.19	3.63	8.05	0.58	
Field	13	2.16	0.23	0.23	0.00	

Table V below shows that the flea index of bandicoots is about four times that of *R. rattus*. This may be due to the greater size and weight of the bandicoot. The field rats are comparatively free from flea infestation.

TABLE V  
*The flea indices for bandicoots, field rats, etc.*

	Bandicoots	House mice	Musk rats	Field rats
Number obtained	17	16	2	13
Total flea index	19.60	2.12	1.00	0.23
<i>X. astia</i> index	18.30	1.00	0.50	0.23
<i>X. cheopis</i> index	1.30	1.12	0.50	0.00

Table VI below shows the close association between the present *cheopis* index and the plague deaths per mille per year for the period 1920—28, in Theni and Uthamapalayam.

TABLE VI

Name of place	Population	Present <i>cheopis</i> index (1928)	Deaths from plague per mille per year for the period 1920—28
<i>Kambam Valley</i> Theni (Allinagaram union)	7,603	2.00	2.1
Uthamapalayam	9,357	4.69	4.5

No such association was observed in Periakulam and Dindigul. The *cheopis* index is high in Dindigul, yet there has been no plague for the last 8 years.

TABLE VII

*Percentage and sex distribution of the Xenopsylla fleas, in the different places*

Places	Total fleas	Percentage of <i>X. astia</i>	Percentage of <i>X. cheopis</i>	Percentage of female <i>X. astia</i> to total <i>X. astia</i>	Percentage of female <i>X. cheopis</i> to total <i>X. cheopis</i>
Periakulam	1,609	75.32	20.13	60.59	50.00
Theni	1,405	70.10	28.82	51.17	42.22
Uthamapalayam	1,906	28.12	57.34	58.39	35.95
Dindigul	1,221	67.00	33.00	50.97	30.84

*Echidnophaga gallinacea* or the fowl fleas were mostly collected from the rats got from the villages. This flea was always found around the mouth, eyes and ears of the rat, just as they are seen on fowls and they actually bury themselves in the tissues of the host.

One *Ctenocephalus canis* was also got from a rat, at Dindigul.

#### OBSERVATIONS

(1) It was observed that cotton mills situated within the town, at Theni and Dindigul, showed a much higher figure for rat population and a higher percentage of *chicopsis* fleas, than the average figures for the town.

Places	Town		Cotton Mills	
	Rats for 100 traps set	Percentage of <i>chicopsis</i> fleas	Rats for 100 traps set	Percentage of <i>chicopsis</i> fleas
Theni	1385	28.82	5300	93.22
Dindigul	1389	33.00	2330	55.26

This fact is of importance and it agrees with observations made in other places that cotton factories are sources of potential danger in addition to grain godowns.

(2) A series of experiments were made to find the average time taken for killing rats and fleas, by simple exposure to the sun. The traps with rats were kept covered in thin cotton cloth bags (white) during the time of exposure. It was uniformly observed in all experiments that the fleas escaped from the body of the rats when exposed to the sun, and that fleas require longer time to die than their hosts. When the average temperature of exposure was 85°F the rats died in 3 hours and the fleas in 4—5 hours. When the average temperatures of exposure were 80°F and below, neither the rats nor the fleas died by exposing them to the sun all the day.

The conditions of this experiment roughly correspond to the disinfection of a bag of rat and flea infested grain, by exposing the bag to the sun, without spreading the grain on any specially prepared platforms. The results show that such methods of sun-disinfection cannot be efficient in places with low temperatures.

(3) The rate of multiplication of rats, as judged by the percentage of pregnant females in a locality, shows a preferential increase in highly insanitary places where prevailing conditions are favourable to rat-life.

#### CONCLUSIONS

(1) The insanitary conditions prevailing in villages in the Kambam Valley, favour a large rat population.

(2) The chief rat-fleas in the areas surveyed are *X astia* and *X cheopis*. In Uthamapalayam and surrounding villages (forming the middle of the Kambam Valley) *X cheopis* is the preponderating flea on rats.

(3) There is very little importation of grains into the Kambam Valley.

(4) The mean daily temperature of the Kambam Valley is below 80°F, the mean saturation deficiency below 0.3 inch, and the available rainfall distributed all through the year.

Plague has appeared almost yearly since 1918, in the Kambam Valley.

The above four conclusions indicate the reasons for the endemicity of plague in this valley, and they further show that probably the epizootics have carried over from year to year.

To sum up, one finds in these villages in the Kambam Valley a set of factors such as high *cheopis* index, frequent and unrestricted movement of grains, bad sanitation, abundance of rats, and ideal climatic conditions. But there are several encouraging features, such as an attempt at improvement of sanitation by the introduction of pit-system of latines, providing zinc-roofing for houses and willingness of the masses for protective inoculation. In course of time when more vigorous preventive measures are concentrated on these villages, it may be possible to stamp out plague from the Kambam Valley, which now appears to be the nursery of this pestilence in the district.

My thanks are due to Lieut-Col H. H. King, I.M.S., Director, King Institute, Gundy, for his kind supervision and suggestions during the survey and for help in writing this report.

# THE INFECTION OF HAMSTERS WITH KALA-AZAR BY THE ORAL ROUTE

BY

MAJOR H E SHORTT, F Z S, I M S,  
MAJOR A C CRAIGHEAD, I M S,  
ASSISTANT SURGEON R O A SMITH, D T M, I M D,  
AND  
C S SWAMINATH,  
*Kala-azar Commission*

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IN a previous publication (Shortt, Craighead, Smith and Swaminath, 1928) we recorded the infection of a hamster, *Cricetulus griseus*, with kala-azar after the administration of infective material by the oral route in the same account a short resume was given of other successful experiments in which infections with kala-azar had been produced via the intestinal tract

As these experiments related in each instance only to one or, at most, two animals, they might have been considered as somewhat exceptional cases and it was obviously important to determine whether infection via the intestinal canal could be readily produced or was a rare phenomenon To settle this point we decided to repeat our experiment previously referred to, using as large a number of animals as was at our disposal and, in addition, to perform a somewhat similar experiment with the flagellate form of *L. donovani*

## *Experiment I*

On 19th April 1928 nineteen hamsters (*Cricetulus griseus*) were given by the mouth about 0.1 to 0.15 c.c. of emulsion in normal saline solution of mixed liver and spleen from a hamster infected with kala-azar The dose in each case was dropped into the hamster's mouth from a pipette without the latter touching the animal The animals were observed to swallow at least a part of the material although a certain amount was wasted

Ten of these animals died next day owing to accidental exposure by an attendant to a hot sun on their way down to the field laboratory of the Kala-azar Commission at Gauhati from Shillong where the experiment had been performed. On 24th April, 1928, one hamster was given by the same method about 0.3 c.c. of saline suspension of the material obtained by liver puncture of a kala-azar patient. None of these animals were given any further doses of any kind and were merely tended carefully during the duration of the experiment.

Of these animals two died on 13th January, 1929. One of these was infected with *L. donovani* and in the other no parasites were found. As both animals showed commencing putrefaction it is quite probable that the second may also have been infected. Another animal died on 26th February, 1929, and proved on examination to be very heavily infected.

The remaining seven animals were sacrificed on 20th March, 1929. Of these six were found to be infected, four heavily, one moderately, and one lightly. To summarize the results, we may state that out of ten hamsters given one oral dose of liver or spleen emulsion, or both mixed, eight became infected, in most cases heavily. Of the two which showed no infection, one had either never become infected or had lost the infection by the time it was examined, while the other was in too putrefied a condition for us to give any certain opinion upon.

### Experiment II

In this experiment six hamsters (*Cricetulus griseus*) were given cultures of *L. donovani* in NNN medium by the oral route. The technique adopted in administering the cultures was the same as that used in the previous experiment. The cultures varied in age from 8 to 22 days old and were administered at intervals of a few days as detailed below —

The fluid from six tubes of NNN medium was centrifuged and the deposit of *L. donovani* was distributed in approximately equal doses to the six hamsters. The details and results of this experiment are given in tabular form below —

Experimental animal	Date of commencement of experiment	Method and date of termination of experiment	Number of oral administrations of culture	Result
Hamster B 1	9th Feb 1928	Found dead, 18th Feb 1929	63	Positive
" B 2	"	Found dead, 16th Feb 1929	60	"
" B 3	"	Sacrificed 20th March 1929	63	"
" B 4	"	Found dead, 16th Feb 1929	57	"
" B 5	"	Sacrificed, 21st March 1929	88	Negative
" B 6	"	Sacrificed 21st March 1929	65	Positive, very heavy infection

In the case of animals which die before they are examined the presence of moderate infections is likely to be missed and light infections are almost certain to be so. If decomposition is advanced, often a matter of only a few hours in the tropics, one may not be able to give a confident positive pronouncement even in the case of heavy infections.

In two out of the three animals found dead,  $B_1$  and  $B_2$ , the spleen was enlarged to three or four times the normal size and in the third,  $B_3$ , to twice the normal size. In hamster  $B_4$ , which showed a very heavy infection, the spleen was enlarged to about twice the normal size. In the remaining animals,  $B_5$  and  $B_6$ , the spleen was not enlarged. We may say, then, that a minimum of three hamsters, out of the six, became infected as the result of oral administration of cultural forms of *L. donovani*.

### Discussion

It is well known that the flagellate form of *L. donovani*, as seen in cultures, will not long survive the introduction of contaminating bacteria. This is also true of the similar forms found in *P. argentipes*. Moreover, if the aflagellate Leishman-Donovan body be introduced with bacteria into NNN medium the bacterial growth inhibits the normal development of the L. D. bodies into flagellate forms and their subsequent multiplication. These facts seemed to rule out the possibility of any form of *L. donovani* introduced into so apparently hostile a habitat as the mammalian intestine existing there for any length of time.

The fact that infections have actually been produced in a large proportion of cases, both with the aflagellate and the flagellate forms of *L. donovani*, via the intestinal canal, nullifies thus *a priori* assumption and indicates that *L. donovani* in either of its forms possesses a degree of resistance to the hostile environment of the mammalian intestine sufficiently great and prolonged to enable it to reach a sterile shelter in the internal organs of the mammal, via the general circulation. The method by which this entry is effected is still to find.

Once this admission is made, however, it at once re-opens the hypothesis of oral infection in kala-azar. In face of the strong circumstantial evidence tending to incriminate *P. argentipes* as the vector of Indian kala-azar, it is with some reluctance that we re-open the question, but the experiments here described leave us no option and render necessary a re-examination of the possibilities of oral infection. This necessity is emphasized by the fact that, as pointed out by us in the publication already referred to, while all attempts to transmit kala-azar by the bites of insects (out of thousands of experiments) have been unsuccessful, yet the relatively very few attempts at infection by the oral route have shown a high percentage of successes. We know that the parasites of kala-azar escape in the urine in viable form and it is most probable that they also escape in the faeces. The obvious line of future experimentation, therefore, is to determine the avenues from which viable forms of *L. donovani* may leave the mammalian host and how far these forms, which show considerable powers of resistance in the mammalian gut, will

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exist in viable form in various food materials such as milk. Even a limited power of resisting dissolution in such a medium might ensure their gaining entrance to the alimentary canal of another host.

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### *CORRIGENDA*

In the October 1929 (Vol XVII, No 2) issue of the *Indian Journal of Medical Research*, please *note* the following corrections —

On page 622, footnote line 1, in place of 'page 623' *read* 'page 617'

„ „ 644, line 7 from bottom, in place of '(See Plate XLIX)' *read*  
'accompanying his paper'



# SEASONAL VARIATIONS IN THE ALKALOIDAL CONTENT OF THE INDIAN EPHEDRA

BY

LIEUT-COL R N CHOPRA, M A, M D (Cantab), I M S,  
*In-charge, Indigenous Drugs Inquiry, I R F Association,*

AND

In  
Medica ASHUTOSH DUTT, B SC  
Or the Department of Pharmacology and Chemistry, Calcutta School of  
Tropical Medicine and Hygiene )

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LIU (1927), discussing the geographical distribution of the plant Ephedra  
*E vulgaris* Rich occurring in the temperate and alpine Himalayas  
at an altitude of 7,000 to 12,000 feet above the sea-level. He also mentions  
*E intermedia* Schrenk et C A Mey var *glauca* Stapf occurring in Tibet and  
adjoining parts of Kashmere. In a previous paper (1928), it was pointed out  
that several species of this plant grow in India. The two common species  
occurring in the Himalayas are *E vulgaris* Rich and *E intermedia* Schrenk and  
Mey, also known as *E pachyclada*, Boiss. A variety of it *E intermedia* var  
*tibetica* also grows in certain parts of Kashmere. Besides these, three other  
varieties are said to grow in India, i.e., *E peduncularis* also known as *E alte*,  
*E fragilis* and *E foliata*, Boiss, to none of which are attributed any medicinal  
properties. We have only been able to examine the last named of these and find  
that it has no alkaloidal content at all. The following table gives the results of

various specimens analysed by us with their total alkaloidal contents and relative quantities of ephedrine and pseudo-ephedrine —

TABLE I

Species	Sources	Total alkaloid per cent	Ephedrine per cent	Pseudo-ephedrine By diff per cent	REMARKS
1 <i>E. vulgaris</i>	Lahoul near Simla	0.93	0.66	0.27	Supplied by Conservator of Forests, Kashmere
Do	Kargil (Kashmere)	1.03	0.72	0.31	
3 Do	Rampur (Jhelum valley)	0.93 to 1.002	0.49 to 0.807	0.348 to 0.36	
4 Do	Forest Department Dehra Dun	0.03			Do
<i>E. pachyclada</i> or <i>intermedia</i> ( <i>tibetica</i> )	Dehra Dun	1.8 (59 per cent ephedrine)	1.06	0.73	Supplied by Mr B. L. Gupta, Dehra Dun
6 <i>Ephedra foliata</i>	Do				Supplied by Mr R. N. Parker, Forest Botanist

A perusal of Table I shows that on the whole the percentage of pseudo-ephedrine is higher in the Indian varieties than in the Chinese. In *E. intermedia* var. *tibetica* it is nearly half of the total alkaloids. The total alkaloidal content of Chinese *Ephedra* varies from 0.018 to 1.32 per cent, of which 10 to 25 per cent is pseudo-ephedrine (the maximum pseudo-ephedrine content being 25 per cent of the total alkaloids) —

TABLE II

Species	Total alkaloids per cent	Ephedrine per cent	Pseudo-ephedrine per cent	REMARKS
Chinese <i>Ephedra</i>	0.018 to 1.32	0.015 to 1.00	0.003 to 0.33	Pseudo-ephedrine in Chinese varieties varies from 10 to 25 per cent of the total alkaloids

It will also be seen that there are considerable variations in the total alkaloidal contents of the specimens of the same species obtained from different localities. This may be due to (1) the difference in the soil and climatic condition of the locality from which the specimen has been collected, and (2) the time of the year at which the collection has been made. The first of these factors is still under

investigation but the seasonal variations we have been able to study with the help and co-operation of Mr S N Kaul, Conservator of Forests, Utilization Division, Kashmir, who was kind enough to obtain for us samples of *E vulgaris* growing in the mountain ranges bordering on the Jhelum valley. These samples were collected from May to December, after that it was not found possible to obtain them on account of heavy snow fall in these hills. The following table gives the results of the alkaloidal contents of different specimens collected —

TABLE III

Time of collection	Total alkaloidal content per cent	Ephedrine	Pseudo-ephedrine By diff
May 1928	0.96	0.61 (64 per cent)	0.35
June ,	0.979		
August ,	0.776	0.428 (55 per cent)	0.348
October	1.17	0.807	0.363
December „	1.002		

A perusal of the table will show that the amount of the total alkaloids gradually increases during the summer months but falls somewhat during the rainy season. It goes up again and attains its maximum in the autumn and then it begins to fall again. The range of variations of total alkaloid is between from 0.77 to 1.17, i.e., about 0.4 per cent and that of ephedrine is from 0.428 to 0.807. The percentage of pseudo-ephedrine does not show very marked variations. These results correspond with those obtained by Feng and Read with *E. equisetina* collected in China.

## SUMMARY AND CONCLUSIONS

- (1) The results of analysis of six specimens of different varieties of ephedra obtained from different localities in India is given.
- (2) The percentage of pseudo-ephedrine appears to be somewhat higher in the Indian varieties as compared with the Chinese varieties.
- (3) Seasonal variations in the alkaloidal contents were worked out from May to December for *E. vulgaris*. The alkaloids gradually increase in the summer month, but appear to fall somewhat in the rainy season. The maximum is attained in the autumn (October) and then there is decline.

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# STUDIES ON THE ENLARGED MALARIAL SPLEEN

## Part I

### EFFECT OF ADRENALIN ON THE BLOOD PICTURE

BY

MAJOR T A HUGHES, M A, M D, M R C P, D P H, I M S,

AND

D L SHRIVASTAVA, D S C

*(From the Department of Clinical Medicine of the King Edward Medical College,  
Lahore )*

[Received for publication, June 14, 1929]

DURING recent years several observers, led by Barcroft and his colleagues (1923 to 1928), have established the fact that the normal spleen contains a reserve of red blood cells which can be rapidly added to the circulating blood and stored away again according to requirements. Among the workers in this field may be mentioned Cruickshank (1926), Hargis and Mann (1925), de Boer and Carroll (1924) and Hanak and Harkavy (1924). It is now known that the spleen is much larger during life than after death, that this organ has the power to concentrate blood and that contraction of the smooth muscle of the spleen, causing expulsion of erythrocytes into the general circulation, occurs under many circumstances. Among such circumstances are hæmorrhage, CO-poisoning, varieties of asphyxia, muscular exercise, pregnancy, 'heat' and injections of adrenalin and pituitrin. de Boer and Carroll showed that in CO-poisoning the change in the volume of the spleen is brought about by stimulation of the spinal cord by oxygen-want, and it is now accepted that the smooth muscle of the spleen contracts in response to stimuli reaching it through the splenic nerves or to an increased output of adrenalin. Thus Isquierdo and Cannon (1928) found that emotional polycythæmia in cats is caused by increased activity of the suprarenal glands, the red cell count reaching its maximum very soon (1 minute) after excitement and returning to the pre-excitement level in thirty minutes.

The relation of the spleen to the white corpuscles differs from its relation to the erythrocytes. While during adult life the latter are produced exclusively in the bone marrow, the mononuclear white cells, viz., lymphocytes and monocytes originate to a large extent in the spleen. Harvey (1906) found that a lymphocytosis followed the injection of pilocarpine, adrenalin, muscarin, and barium chloride, and came to the conclusion that these drugs caused a contraction of the spleen and lymph glands. Menkin (1928), working on cats, showed that emotional excitement brought about an increase of 13 per cent in the total mononuclear cells of the blood. The increase was maintained for 10 to 15 minutes after the period of excitement and the normal was reached in 20 to 30 minutes. This phenomenon did not occur in splenectomized or sympathectomized animals, and was therefore due to contraction of the spleen.

This paper contains a record of experiments made to determine the changes in the blood picture brought about by subcutaneous injection of adrenalin in patients having enlarged spleens, the result of chronic malaria. All subjects examined had an increase in the circulating monocytes. In this connection it should be noted that a relative monocytosis is often found in persons whose spleens are not appreciably enlarged and who show no signs of active malaria. This may be due to a small latent infection or may possibly be the result of malaria which has been eradicated. This circumstance rendered difficult the finding of strictly normal controls as nearly all the individuals available had suffered from malaria at some time or other.

#### EXPERIMENTAL

In each experiment a red cell count and a total and differential leucocyte count were done on a patient before and after the subcutaneous injection of 1 c.c. of adrenalin, 1 in 1000 (P. D. & Co.). Preliminary observations were made to determine the time after injection at which the blood changes were most marked. It was found that the red cells reached their maximum 25 minutes, and the white cells 20 minutes after adrenalin. Subsequently all counts were made at these intervals. Haemoglobin estimations were found to be unsatisfactory owing to the difficulty in detecting small changes by the methods at our disposal, especially in anæmic patients. Films were stained by Giemsa's method. In the differential counts the numbers of small and large lymphocytes and of monocytes are given separately, although it is recognized that the differentiation of large lymphocytes from monocytes is sometimes very difficult. Distinction was based on the descriptions given by Piney (1928), having special regard to the nuclear basi-chromatin. Cells with a linear arrangement of this substance were classed as monocytes while nuclei showing dense masses of basi-chromatin were taken as belonging to large lymphocytes. When any doubt arose the benefit was given to the lymphocytes, so as not to over-emphasize the mononuclear increase, especially in post-adrenalin counts. In every experiment at least 300 cells were counted before and after injection.







Results are set forth in Tables I and II. In Table I absolute figures are given and in Table II percentage changes. Observations 1, 2 and 3 were made on normals. On each of four patients, two experiments were carried out on different dates. In seven cases there was little or no rise in the red blood cells, while in 3 the increase was of the same order as in the controls. Of these three

TABLE II

*Showing the percentage changes in blood counts after Adrenalin injection*  
(The numbers refer to those of patients given in Table I)

No	R B C	W B C	Large lymphocytes	Small lymphocytes	Monocytes	Polynuclears
1	+22.0	+36.6	+14.0	+13.1	+77.0	+37.7
2	+18.5	+21.2	+34.2	+21.3	+37.4	+16.6
3	+22.6	+75.9	+99.2	+70.5	+102.4	+67.9
4	+24.7	+15.7	+81.8	+26.2	+35.8	-7.9
5	+20.2	+84.6	+322.5	+93.0	+70.5	+51.5
6	+23.0	+11.7	+77.3	+29.8	-37.1	+12.3
	+15.4	+37.9	+18.2	-58.6	+156.2	+29.0
7	+3.1	+53.3	+79.5	+67.8	-5.6	+63.0
		+41.8	+81.1	+18.0	+85.6	+75.9
8	+6.7	+142.5	+324.5	+6.7	+270.1	+81.0
	-0.3	+136.3	+136.2	+2.7	+184.7	+215.0
9	+5.2	+88.0	+208.5	-6.0	+155.6	+41.5
10	+5.4	+35.4	+108.3	+17.9	+88.4	+21.1
11	+2.4	+105.3	+118.1	+96.7	+190.2	+97.6
12	+0.6	+60.7	+208.0	+26.7	+156.0	+21.8
13	+0.2	+64.3	+93.8	-6.3	+154.0	+45.3

cases one (No 4) was suffering from tubercular disease of the small intestine, and one (No 6) from an acute exacerbation of malaria (mixed B T and S T infection). In only one experiment was there a fall in the red cells, and this was insignificant. An increase in the total leucocyte count occurred in every case, and while there is no constant relationship between the rise in white cells and that in reds, it is seen that the patients showing the greatest degree of leucocytosis are amongst those in whom there was little or no change in the erythrocyte count. The increase is greatest and most constant in the case of the large lymphocytes and monocytes, i.e., the 'large mononuclears,' and here again the most

marked changes are accompanied by little alteration in the number of red cells. In two experiments there was a fall in the monocytes after adrenalin. These experiments were carried out on patients who were at the time suffering from acute attacks of malaria. Examination of the same patients after a few days of intensive quinine treatment revealed a post-adrenalin rise in monocytes. Variations in the granulocytes were irregular, as were also the changes in small lymphocytes. Attention may be directed to the high counts in patient No. 11 who was suffering from influenzal broncho-pneumonia. These are most likely due to concentration of the blood, a phenomenon which is well known to occur in this disease.

### DISCUSSION

Our results show that the enlarged spleen in chronic malaria may contain little or no reserve of red blood cells, especially in the absence of an acute condition. The outstanding histological features of the chronic malarial spleen, apart from the presence of parasites and of malarial and blood-derived pigments, is the proliferation of the cells belonging to the reticulo-endothelial system. These cells invade the blood sinuses and diminish the capacity of the organ to store red corpuscles. Contraction of such a spleen would cause a rise in the numbers of circulating lymphocytes and monocytes. In all our experiments except two we found this to be the case, and it was always observed that after adrenalin the blood contained an increased proportion of mononuclear cells of a large type, such as are seen in the spleen, liver and bone marrow when the reticulo-endothelial cells are stimulated to proliferation (Gye and Purdy, 1922, 1924).

Of the two patients who showed a fall in the monocytes after adrenalin when first examined, one had a very heavy double infection which had been rapidly getting worse without treatment. In the other case there was a severe subtertian infection. This patient was a cooly of very poor physique and under-nourished, and had comparatively little enlargement of the spleen. When the disease had been got under, by means of quinine, the number of circulating monocytes diminished considerably in both patients, but there was a large output of these cells on stimulation of the spleen. The findings in these two cases strengthen the impression obtained from the general results that the reticulo-endothelial cells of the spleen play an important part in overcoming malarial infection.

### CONCLUSIONS

(1) Observations were made on the changes in the blood picture brought about by contraction of the enlarged spleen in chronic malarial patients following the subcutaneous injection of 1 c.c. adrenalin solution (1 in 1000).

(2) In some patients of this type contraction of the spleen causes little or no increase in the circulating red cells, especially in the absence of an acute condition.

(3) A leucocytosis always followed injection of adrenalin, the most marked change in quiescent cases being an increase in the monocytes and large lymphocytes.

Our thanks are due to Dr Lal Chand Khanna, Professor of Physiology, King Edward Medical College, Lahore, for permission to work in his Laboratory and to Dr Aman Ullah Khan for assistance during the investigation.

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# STUDIES ON THE ENLARGED MALARIAL SPLEEN

## Part II

### EFFECTS OF INTRAVENOUS AND ORAL ADMINISTRATION OF QUININE ON THE BLOOD PICTURE

BY

MAJOR T. A. HUGHES, M.A., M.D., M.R.C.P., D.P.H., I.M.S.,

AND

D. L. SHRIVASTAVA, D.Sc.

*(From the Department of Clinical Medicine King Edward Medical College,  
Lahore)*

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It has been shown in a previous communication (Hughes and Shrivastava, 1930) that contraction by adrenalin of the enlarged spleen of chronic malarial patients, not suffering from any acute condition, brings about an increase in the circulating leucocytes especially of the monocytes and large lymphocytes, but that the capacity of such a spleen to act as a reservoir for red blood cells may be much reduced or totally absent. In this paper are recorded the results of an investigation into the changes in the blood picture caused by quinine in similar subjects and in malarial patients with no appreciable splenic enlargement. Two patients were suffering from post-malarial cirrhosis of the liver.

The method by which quinine destroys malarial parasites is not known, but its action is undoubtedly an indirect one. Yorke (1926) believes that large numbers of parasites are first killed, probably indirectly, and that these act as antigen and call forth an output of immune bodies from the tissue cells. Mancy (1928) reviews the whole subject and quotes Kirschbaum (1923) who found that parasites remained alive for six hours outside the body in blood containing quinine in a much greater strength than is found in the circulating blood after oral or intravenous administration. Giemsa (1927) is of opinion that quinine is absorbed by the endothelial cells and given up to the parasites, while Voinik (1927) considers that the drug lyses the red blood cells containing parasites which are set

( 657 )

free in the plasma and there destroyed Cushny (1926), however, states that quinine causes hæmolysis only when it is present in the blood to the extent of 0.5 per cent, a strength which is more than sufficient to stop the heart. To reconcile these statements, it is necessary to assume that corpuscles infected with parasites are more vulnerable to quinine than normal red cells. Voinik states that in malaria the hæmolytic action of quinine varies inversely as the NaCl content of the blood, and that an elevated temperature increases hæmolysis. As regards the effect of the drug on the spleen Cushny quotes Roth's opinion that 'a single dose generally causes leucocytosis at first, perhaps arising from contraction of the spleen. This is followed by a fall in the number of white corpuscles, especially of the lymphocytes, though the polynuclears are also reduced.'

### EXPERIMENTAL

The enquiry was carried out on the lines already described (Hughes and Shrivastava, 1930). In one set of experiments a single dose of quinine hydrochloride (6 grains) was given intravenously, while in another each patient received 10 grains of quinine hydrochloride in solution four times a day, by the mouth. The effect of quinine and alkalies, given as recommended by Sinton (1923), was also tried. Sometimes this combination was administered after several days of quinine alone, sometimes after several days of alkalies alone. In these observations the urine was kept alkaline throughout.

### RESULTS

Typical results after intravenous quinine are given in Tables I and II, the former showing absolute figures and the latter percentage changes. In the earlier experiments, counts were made 20 minutes after injection. At this period the usual findings in malarial subjects were a fall in the red cells and a rise in the leucocytes, chiefly affecting the large lymphocytes and the monocytes. It was found later that counts made at an interval of one hour usually showed a greater increase in the white cells, while the erythrocytes had returned to the original level or gone above it. Two hours after injection the number of leucocytes was usually below the pre-injection level. Control experiments using saline alone were without effect. In one of the cases of cirrhosis of the liver (No. 6), the changes after intravenous quinine were relatively very slight. When injections were given to individuals without splenic enlargement or other evidence of malaria the changes were a rise in leucocytes as before, accompanied, however, by a rise in the red cells.

During oral administration of quinine, of alkalies and of quinine and alkalies, counts were made at exact intervals of 24 hours, before the morning meal. In some of the early experiments with quinine alone, the blood was examined 20 minutes and 2 hours after the first dose of the drug as well. Typical results of this series are given in Tables III and IV and two experiments with quinine and alkalies are represented graphically in Figures 1 and 2. Figure 1 shows the changes in total leucocytes and in granulocytes, and Figure 2 in large lymphocytes.







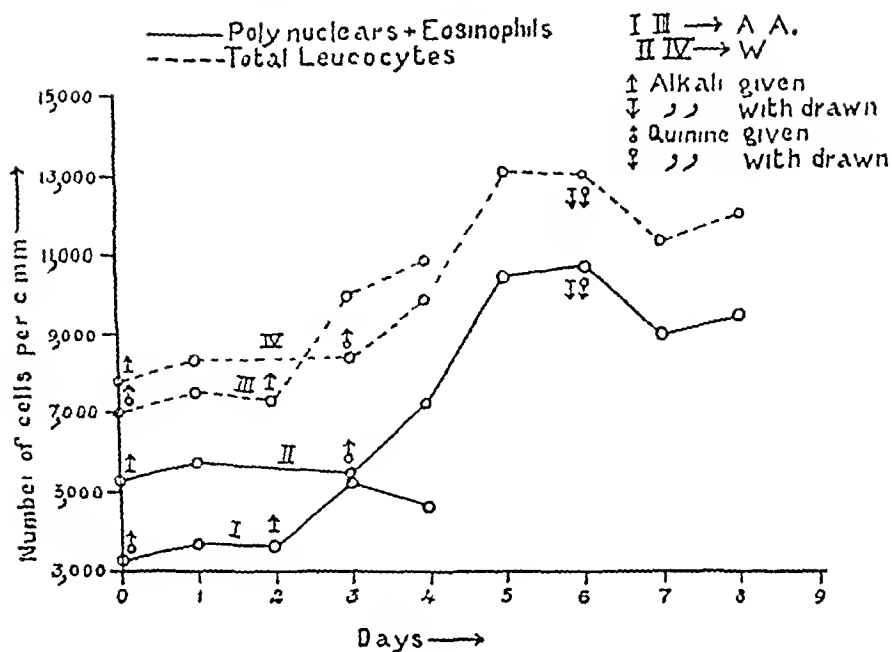


Fig 1

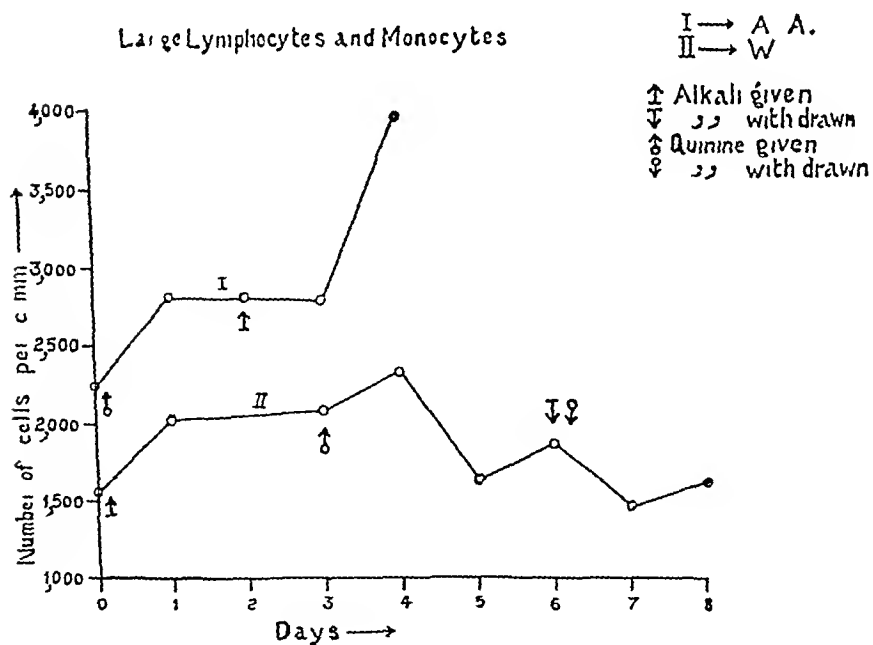


Fig 2

and monocytes combined These two types of cells are lumped together because of the difficulty that sometimes exists in distinguishing one from the other

With oral quinine there was a small increase in the total white cells which affected chiefly the large lymphocytes and monocytes When alkalies were given with quinine a sharp rise above the pre-quinine figures was produced in which, however, the granular cells often took a prominent part (Case W, Table III and figures) This change reached its maximum on the second or third day The red cells did not show any marked changes with quinine alone, but with alkali and

TABLE II

*Percentage changes in blood counts after intravenous injection of quinine*  
(The numbers refer to those of patients given in Table I)

No	R B C	W B C	Large lymphocytes	Small lymphocytes	Mono cytes	Polynuclears	REMARKS
1	-17.4	+41.6	+205.3	-12.8	+244.0	+6.11	
	-22.2	+13.5	+41.1	-40.4	+148.0	+4.7	
2	-7.9	+74.7	+88.9	+22.3	+108.4	+82.5	
3	-21.0	+46.7	+55.8	+122.2	+54.1	+15.2	
	-8.9	+55.5	+50.4	+29.5	+65.2	+58.7	
	-10.8	+31.3	+92.0	+11.1	+59.4	+15.0	
	+5.8	+41.3	+76.6	+56.5	+120.5	+22.1	Changes 1 hour after injection
4	-17.4	+28.4	+28.5	+65.8	+65.4	-3.3	
	-18.5	+14.8	+8.6	-59.4	+222.3	+1.1	
	+6.3	+55.5	+75.9	-39.7	+279.5	+42.4	Changes 1½ hours after injection
5	-16.5	+11.8	-32.0	-66.6	+95.1	+21.6	
6	-3.0	+9.4	-87.1	+46.3	-18.1	+15.2	
7	-21.6	+73.8	+92.2	-38.7	+191.3	+5.8	
8	+9.6	+16.1					
	+11.5	+112.2	+512.9	-9.0	+286.4	+33.5	Changes 1 hour after injection
	+6.2	-3.0	+65.0	-65.4	+32.6	+7.1	Changes 2 hours after injection
	+6.8	+5.5					
9	+11.9	+58.8	-23.7	-30.0	+28.4	+96.4	Changes 1 hour after injection
	+6.6	-19.5					Changes 2 hours after injection

Figures refer to changes occurring 20 minutes after injection unless otherwise stated

TABLE III

Effect of oral quinine on blood count

No	Date	Clinical Notes	R B C per cmm (millions)	W B C per cmm	I rego lympho cytes per cmm	Small lympho cytes per cmm	Mono cytes per cmm	Polynu clears per cmm	Leuko phils per cmm	Bilirubin in units	Remarks
I	8-2-29	A A History of chronic malaria. Spleen 5 inches and liver 1 inch below costal margin	4.62	7,030	551	1,197	1,615	3,163	111		20 minutes after quinine do
	Do		5.01	7,200	576	811	2,282	3,106	122		2 hours
	Do		4.84	7,230	412	131	2,116	4,100	166	Traces	Quinine 10 grains 9th February Do 10th February
	9-2-29	S S History of chronic malaria. Irregular fever, spleen 1 inch below costal margin	4.70	7,600	760	1,061	2,652	3,670	53	Traces	Do + alkalies 11th February Do 12th February
	10-2-29		4.99	7,300	1,040	832	1,771	3,526	124		
II	11-2-29		5.33	9,970	867	1,921	1,924	5,125	130		
	12-2-29	S S History of chronic malaria. Irregular fever, spleen 1 inch below costal margin	5.32	10,830	1,871	2,212	2,090	1,581	11		
	15-2-29		5.93	9,730	1,168	1,722	2,110	1,952	177	1.7	Alkalies
	16-2-29		5.91	9,970	2,123	1,635	1,795	3,059	169		Do
	18-2-29		6.29	9,570	1,550	1,520	1,111	1,816	513	2.8	Alkalies and quinine
III	19-2-29		6.02	11,500	2,150	1,184	2,610	1,861	690	1	Do do
	21-2-29		6.30	12,330	1,887	1,110	2,133	6,375	826	1.7	
	22-2-29	W History of chronic malaria. No obvious enlargement of spleen or liver. Ankylostoma maias	4.84	7,870	708	944	842	1,015	1,361	Nil	
	23-2-29		4.97	8,330	708	525	1,316	1,311	1,157		Alkalies
	25-2-29		4.74	8,470	778	851	1,297	1,452	1,087	Nil	Do
	26-2-29		4.94	9,870	563	306	1,776	6,839	185		Alkalies and quinine
	27-2-29		5.17	13,070	170	1,007	1,477	10,050	110	Do do	
	28-2-29		5.27	12,930	617	127	1,216	10,130	517	Do do	
	1-3-29		5.23	11,230	595	831	865	8,085	851	No alkalies, no quinine	
	2-3-29		5.24	11,970	239	958	1,365	8,810	599	Do do	

TABLE IV

*Percentage changes in blood counts after giving quinine orally**(The numbers refer to those of patients given in Table III)*

No	Date	R B C	W B C	Large lymphocytes	Small lymphocytes	Mono cytes	Polynuclears	REMARKS
I	8-2-29							
	Do	+ 84	+ 24	- 14	-45.6	+ 38.7	+ 7.7	
	Do	+ 48	+ 28	- 29.5	-71.0	+ 28.8	+ 33.0	
	9-2-29	+ 17	+ 81	+ 30.1	-28.9	+ 24.7	+ 16.0	Same as in Table III.
	10-2-29	+ 80	+ 38	+ 88.0	-44.4	+ 7.8	+ 11.5	
	11-2-29	+15.4	+ 4.2	+ 48.5	+28.5	+ 17.4	+ 62.0	
	12-2-29	+15.2	+54.0	+220.9	+49.8	+ 27.0	+ 44.8	
II	15-2-29							
	16-2-29	- 0.3	+ 4.1	+ 80.0	- 5.1	- 16.1	- 6.9	
	18-2-29	+ 6.1	+ 2.4	+ 32.7	-11.7	- 31.3	+ 13.3	Same as in Table III
	19-2-29	+ 1.5	+29.9	+ 84.1	-31.2	+ 21.9	+ 14.4	
	21-2-29	+ 6.2	+43.8	+ 61.6	-34.6	- 0.3	+ 49.4	
III	22-2-29							
	23-2-29	+ 2.7	+ 5.8	<i>Nil</i>	-44.4	+ 56.3	+ 7.9	
	25-2-29	- 2.1	+ 6.4	+ 9.9	- 9.6	+ 54.0	+ 8.4	
	26-2-29	- 2.1	+25.4	- 25.7	-67.6	+110.9	+ 70.3	
	27-2-29	+ 6.8	+66.7	- 26.0	+ 6.7	+ 75.4	+150.3	Same as in Table III
	28-2-29	+ 8.9	+64.3	- 8.6	-54.8	+ 44.3	+152.1	
	1-3-29	+ 8.1	+55.4	- 16.0	-12.0	+ 2.7	+101.4	
	2-3-29	+ 8.4	+52.1	- 66.3	+ 1.4	+ 62.1	+119.5	

quinine there was usually a definite rise. An erythrocyte count made 20 minutes after the initial dose of quinine often revealed a small increase, but after two hours this had usually gone. The rise in red cells and leucocytes following quinine and alkalies continued for at least two days after the drugs were stopped. Alkalies alone produced no constant effect.

## DISCUSSION

The alterations brought about in the picture of normal subjects by an intravenous injection of quinine resemble those which follow an injection of adrenalin (Hughes and Shrivastava, 1930) and are more than likely due to the same cause,

viz., contraction of the spleen. In malarial patients, however, although the white cell changes after intravenous quinine are similar to those after adrenalin, the alteration in the red cells is different. Here the usual effect is a fall soon after injection, succeeded by a return to normal or a rise in the course of an hour or so. The cause of this fall is, we think, revealed in the increase of plasma bilirubin which we found to occur in most of these cases some time after the administration of quinine. An excessive degree of hæmolysis is suddenly brought about and bile pigment is produced in increased amount by the cells of the reticulo-endothelial system. A temporary hyper-bilirubinaemia consequently results in those individuals whose livers cannot excrete the pigment as fast as it is formed. The formation of bilirubin is a relatively slow process. Mann and his colleagues (1926) found that when hæmoglobin was perfused through the spleen in dogs, the bilirubin content of the blood in the splenic vein went on increasing for 1 or 2 hours. A rise was usually first noticed in 30 minutes. In our cases a significant change in the plasma bilirubin was not as a rule found before one hour, sometimes much later (*cf.* Case 7). A rise in the bilirubin content of the blood would also result from damage to the liver cells such as has been found by Siperstein and Litman (1921) and others to occur in rabbits after quinine administration. Interference with liver function would not, however, cause a fall in the red corpuscles. Further, in normals, in whom a rise and not a fall in red cells resulted, there was no increase in plasma bilirubin. This last fact also suggests that the corpuscles destroyed by quinine are either infected with malarial parasites or are otherwise rendered more vulnerable to the drug by the disease. The subsequent rise in red cells is, we think, due to the splenic contraction persisting after the hæmolytic action has passed off.

During oral administration the blood changes were, as might be expected, different from those that followed a single intravenous injection. In the former case the drug is slowly and continuously entering the circulation over long periods and is passing through the liver where, according to Lipkin (1919), it is acted on by a quinine-destroying agent. After intravenous administration, on the other hand, there is for a short time in the blood a relatively high concentration of quinine which, however, soon falls.

As far as the white cells are concerned, the changes were in the same direction with oral as with intravenous quinine but were much less in degree. There was in no case a significant fall in the red cells, nor was there an appreciable rise in the plasma bilirubin. These facts do not necessarily rule out any destruction of erythrocytes, as the numbers of cells destroyed may have been compensated for by additions from the spleen or marrow, while the liver may have been able to remove any extra bile pigment as fast as it was being formed. While it is reasonable to assume that contraction of the spleen occurs after oral as well as after intravenous quinine, though to a less extent, it is not improbable that in experiments such as these, extending over days, new formation of blood cells contributes to the changes observed. If, as Giemsa thinks, quinine is taken up by the cells of the reticulo-endothelial system, a stimulus to the proliferation

of these cells at least is present during absorption of the drug. On the administration of quinine with alkalis the increase, in some cases, in both granulocytes and erythrocytes suggests increased marrow function. This combination of drugs, however, reduces the size of the enlarged malarial spleen more rapidly than quinine alone (Sinton, 1929), so that shrinkage of this organ must play an important part in the causation of the blood changes. The accentuation by alkalis of the effect of quinine on the spleen is probably connected with the increased tonicity of smooth muscle caused by a rise in the pH (McDowall, 1928). This would sensitize the contractile elements of the spleen to the action of quinine whether this is brought about through the splenic nerves or through an augmentation of the adrenalin output. Menkin (1929) working on rabbits has very recently shown that 'the spleen, while contracting, is able to change the differential leucocyte formula, first by discharging primarily mononuclear cells from its pulp, and secondly, in the course of continued contraction by lowering the absolute leucocyte level in the circulation through an unequal retention, involving the polymorphonuclears to a larger extent than the mononuclears'. This observer had previously (1928) noticed that in splenectomized or sympathectomized cats, prolonged excitement produced a relative decrease of the mononuclears in the peripheral blood. He now attributes this phenomenon to an increase in polynuclears most likely from the bone marrow owing to the fact that these cells could not be retained in a splenectomized or in a sympathectomized animal where the spleen could not contract. It is possible that prolonged intense contraction of the malarial spleen by quinine and alkalis leads at first to an output of mononuclear, and a retention of polynuclear cells as in Case 1, Table III (Fig 1), and that later, when the organ has contracted to its utmost, polynuclear cells coming from the bone marrow can no longer be retained, and consequently increase in number for a time in the peripheral blood (Case 3, Table III, Fig 2).

### CONCLUSION

A study was made of the changes in the blood picture in chronic malarial patients with enlarged spleens following the administration of quinine (a) by the intravenous, and (b) by the oral route.

(a) Intravenous injection of 6 grains of quinine hydrochloride in 10 cc saline brought about the following changes —

- 1 An increase in the leucocytes mostly affecting the large lymphocytes and monocytes. This is similar to the leucocytosis that follows injection of adrenalin in such subjects.

- 2 A fall in the red cells succeeded by a return to normal or a slight increase.

There was also a delayed increase in the bilirubin content of the plasma. Reasons are given for considering this to be an indication of hæmolysis. It did not occur in control experiments on normal subjects.



(b) Blood counts made at intervals of 24 hours on patients having 10 grains of quinine hydrochloride by the mouth four times a day revealed the following changes —

- 1 A rise in the leucocytes always affecting the monocytes
- 2 A small rise in the red cells

When quinine in these doses was combined with alkalies there was a sharp rise in both white and red cells reaching a maximum about the second day. The granulocytes often took a prominent part in the leucocytosis.

Our best thanks are due to Dr Lal Chand Khanna, Professor of Physiology, King Edward Medical College, Lahore, for permission to work in his Laboratory and to Dr Aman Ullah Khan for assistance during the investigation.

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\* Seen in abstract only



# WHOLE WHEAT BREAD AND WHITE BREAD A COMPARATIVE STUDY

BY

BRIANT-COLONEL R McCARRISON, CIL, KHP, MD, FRCP, IMS,  
*Director, Nutritional Research, I R F A, Pasteur Institute, Coonoor, S India*

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## Introduction

THE wheat grain consists of the endosperm, the embryo plant or germ and the pericarp and integuments or outmost layers of the seed. The endosperm, of which white flour mainly consists, constitutes about 83 parts of the entire grain, the germ about 15 parts, and the pericarp and integuments (the bran) the remainder. The chief constituents of wheat are carbohydrates (starch and sugar), proteins, fats, inorganic salts and combined phosphorus, vitamins, crude fibre and cellulose. The carbohydrates are contained chiefly in the endosperm, the fats in the germ, and the proteins and inorganic salts in the germ and bran in which also the vitamins, crude fibre and cellulose for the most part reside. In the process of manufacture of white flour the germ, the pericarp and the integuments—technically called 'the offal'—are removed and discarded. Accordingly, much of the fat, the protein, the mineral matter, the vitamins, and crude fibre is lost. Of these losses the most serious are those of proteins, of salts, of vitamins and of crude fibre.

The loss of protein is serious because the proteins of the embryo and bran are of relatively high biological value, resembling more closely the physiologically active proteins of animal tissues (1, 3), they are superior to those of the endosperm (white flour), and more adequately supply the protein requirements of the growing animal than those of the endosperm (2, 3), further, they are of such a character as to supplement nutritively those of the endosperm, being richer in those nutritionally essential amino-acids in which the proteins of the endosperm are deficient (2, 3). Their discard is wasteful because, without them, the proteins of white flour (gliadin and glutenin) cannot be effectually transformed into body-proteins, suitable amino-acids must, therefore, be provided from other (and expensive) sources, such as milk, meat and egg.

The loss of inorganic elements—lime, phosphorus, iron, manganese, alumina, magnesia, potash, soda, sulphur, chlorine and iodine—and of vitamins is no less serious and calls for a wise selection of supplementary food-materials if the

deficiency of white flour in these essential substances is to be made good. The loss of the crude fibre of wheat bran deprives the intestine of an important laxative (?).

Whole wheat flour (*atta*) contains the products of the whole grain with the exception of the coarser parts of the bran which are removed by sifting, and though it is deficient in certain mineral elements—calcium, iron, sodium, phosphorus and chlorine—yet its deficiencies are much less than those of white flour and are correspondingly easier to rectify.

The investigation with which the present paper deals was undertaken with the object of comparing the nutritive values of diets having white flour or white bread as their basis with those of similarly constituted diets having whole wheat flour (*atta*), or the unleavened bread (*chapatti*) made from it, as their basis. Moreover, the experiments were designed to indicate means by which complete diets could be built up from either of these flours or breads.

The first part of the paper deals with a comparative experimental study of white flour and whole wheat flour (*atta*), the second with a similar study of white bread and unleavened bread (*chapatti*) made from whole wheat flour.

## PART I.

### Whole wheat flour *versus* white flour.

#### *Composition of the flours*

The flours used in these experiments were those on sale in the local bazaar. Both were imported from Calcutta and were manufactured from wheat grown in Northern India. Their chemical composition is shown in Table I.

The iodine-content of the two flours was not determined, but whole wheat flour contains approximately 15 parts of iodine per  $10^6$ , white flour approximately 3 parts per  $10^6$ , the former being from five to six times richer in iodine than the latter. It will be noted from Table I that the *atta* contained more fats, proteins, fibre and mineral matter than the white flour. The former was richer in phosphorus, iron, alumina, manganese, lime, magnesia, potash, sulphur, soda and iodine, than the latter.

#### Details of experiments.

Young rats, aged between 40 and 50 days, and weighing between 32 and 55 grammes, were used. They were divided into groups of 6. There were three males and three females in each group. The aggregate weight of the animals in each group was, as far as possible, the same. The animals were selected from a large number of litters, specially bred for the purpose of the experiment, each member of a group being taken from a different litter. In this way any inherited growth tendencies were equally distributed in the different groups. One group was used for each test. Each rat was confined in a separate, screened cage under conditions of the most scrupulous cleanliness. Coprophagy was prevented. Water for drinking and washing purposes—the latter an important point—was supplied in abundance. The animals were weighed weekly.

TABLE I

Giving the chemical composition of the whole wheat and the white flour used in the investigation results expressed in percentages of moisture-free sample

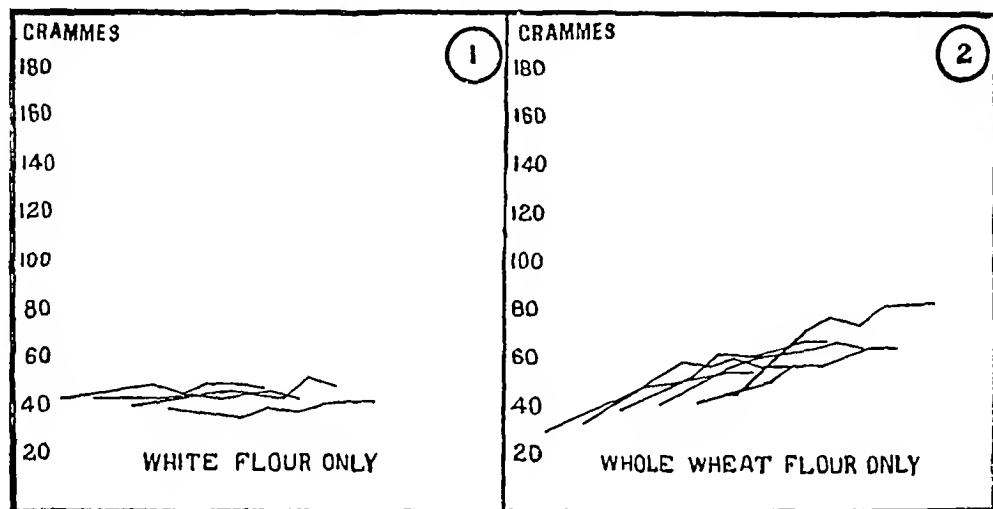
Constituents	Whole wheat flour	White flour
Moisture (110°C)	10.05	11.98
Mineral matter	2.051	0.471
Ether extractives	2.749	1.244
Crude proteins	13.61	9.63
Crude fibre	1.991	0.5
Carbohydrates	79.599	88.155
Total	100.000	100.000
Albuminoids	11.29	8.45
Mineral matter		
Insoluble	0.043	0.016
Soluble	2.008	0.455
Soluble mineral matter		
Soluble silica	Nil	0.002
Phosphoric acid ( $P_2O_5$ )	0.960	0.002
Iron oxide ( $Fe_2O_3$ )	0.04	Trace
Alumina ( $Al_2O_3$ )	0.062	Trace
Manganese ( $Mn_2O_3$ )	0.0086	0.0008
Lime ( $CaO$ )	0.22	0.09
Magnesia ( $MgO$ )	0.247	Trace
Potash ( $K_2O$ )	0.343	0.08
Sulphuric acid ( $SO_3$ )	0.064	0.05
Chlorine (Cl)	Trace	0.018
Soda (by difference)	0.064	0.029
Total	2.008	0.455
TOTAL CHLORINE AS DETERMINED BY THE SOAKING METHOD	0.146	0.138

The experiments were carried out during the spring months, they run concurrently, the experimental period was 54 days

### Series A

#### *I White flour versus 'atta' as the sole article of diet*

The effects of exclusive diets of (a) white flour and water, and (b) *atta* and water on the growth of young rats are shown in Charts 1 and 2



Little growth occurred on the white flour diet, the average increase in body-weight of the four animals that survived the experiment being 10.6 per cent. Considerable growth occurred on the diet of *atta* and water, the average increase in body-weight being 65 per cent, or approximately 55 per cent greater than the growth resulting from the white flour diet. Growth on the *atta* diet was not optimal, though superior to that on the white flour diet.

The white flour diet rarely or never caused polyneuritis, even when the experiment was continued for long periods. Young rats have survived an exclusive diet of white flour, with but little gain in body-weight, for periods as prolonged as 177 days without the development of polyneuritis. It was rare, however, that they survived for so long. Usually they died of pneumonia, gastroenteritis or asthenia with marked alimentary dystrophy. Amongst those surviving for long periods vesical calculus and its sequelæ—cystitis, dilated ureters, pyelitis and hydro- or pyo-nephrosis—were sometimes found at post-mortem examination.

The results of this experiment indicate (a) that while neither whole wheat flour nor white flour is in itself sufficient for optimal growth, neither being a complete food, yet whole wheat flour is more suitably constituted to maintain well-being in young rats for a short time—a conclusion which has already been reached by McCollum and Simmonds(+), (b) that an exclusive diet of the Indian white flour used in this investigation contains just enough of the anti-neuritic fraction of vitamin B (vitamin B<sub>1</sub>) to prevent polyneuritis in growing rats. Some further explanation of the second of these conclusions is necessary.

An experiment was undertaken in which 15 groups of 6 young rats were employed. To one group an exclusive diet of Indian white flour was given, to other groups diets were given in which certain percentages of the flour were replaced by fats, inorganic salts, proteins and purified starch, either alone or in combination. It was found that cases of polyneuritis did not begin to appear until 23 per cent of the white flour had been replaced by other food-materials (5 per cent of salts, 8 per cent of olive oil, and 10 per cent of meat residue) containing little or no vitamin B<sub>1</sub>. Even when these food-materials formed as much as 35 per cent of the diet (20 per cent of meat residue, 10 per cent of olive oil, and 5 per cent of salts) polyneuritis was only an occasional incident during the 65 days the experiment lasted. The further replacement of the remaining 65 per cent of white flour by purified, vitaminless starch caused the incidence of polyneuritis to increase proportionately to the increasing quantity of purified starch in the food. When all the white flour had been replaced by vitaminless starch, polyneuritis was inevitable and caused the death of all animals in periods ranging from 38 to 48 days. The presence of some vitamin B<sub>1</sub> in the Indian white flour was thus conclusively demonstrated.

Rats fed on diets containing from 60 to 77 per cent of white flour, the balance being made up of meat residue, olive oil and salt mixture, occasionally survived for long periods before developing polyneuritis. Examples are shown in Plate I, figs 1 and 2, and Charts 3 and 4. The importance of these observations from the point of view of the causation of beri-beri-like maladies in man

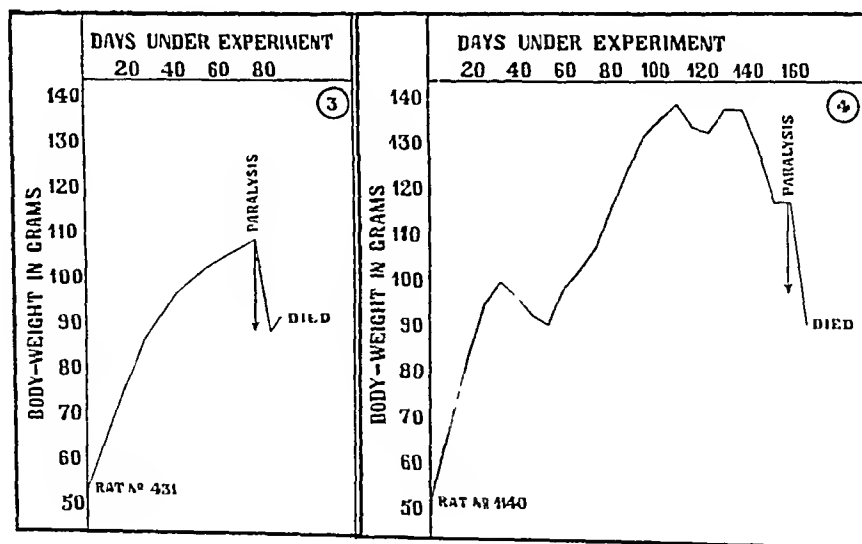


Chart 3—Weight curve of rat No 431 (Fig 1). Body-weight increased steadily up to the 75th day when paralysis occurred thereafter body-weight fell until death occurred on the 86th day.

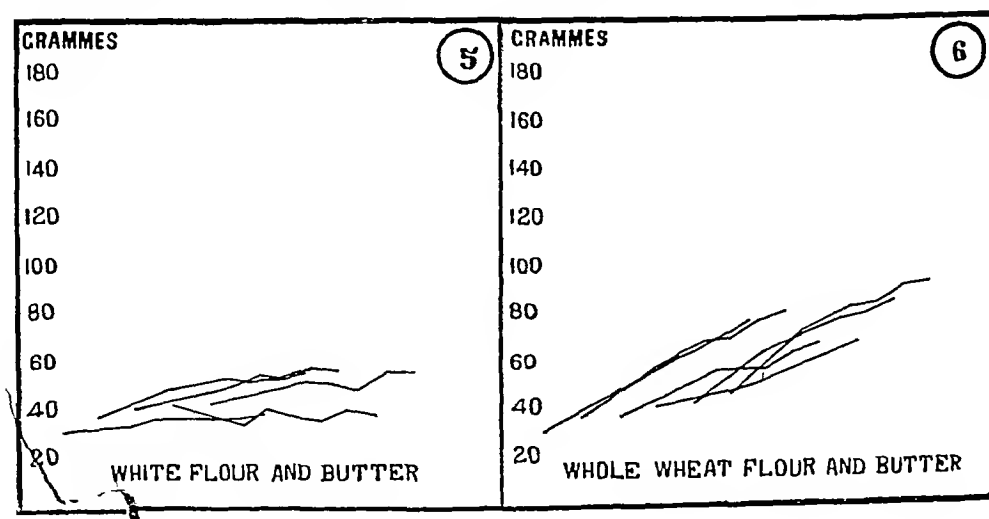
Chart 4—Weight curve of rat No 1140 (Fig 2). Body-weight increased up to 140 grammes on the 108th day thereafter it began to fall, paralysis appeared on the 156th day and death occurred on the 164th day.

is considerable. Beri-beri is not due to complete absence of vitamin B<sub>1</sub>, the dietetic factor in its causation is an insufficiency of vitamin B<sub>1</sub> in the food(5). This insufficiency may be relative to other ingredients of the diet(6). Now white flour contains appreciable amounts of vitamin B<sub>1</sub>, the addition to it of 2 per cent of yeast—the usual amount required for the making of white bread—adds to the supply of vitamin B<sub>1</sub>, consequently, beri-beri is comparatively rare amongst white-bread-eating races. Judging from experience in rats, polyneuritis would be unlikely to arise in persons subsisting on an exclusive diet of white bread and water. But with the substitution of other food-materials (meat, sugar, etc) containing little or no vitamin B<sub>1</sub>, for a considerable proportion of the white bread, cases of polyneuritis or even actual outbreaks of beri-beri-like maladies might be expected to arise under favourable climatic conditions, especially in persons suffering from such tropical infections as malaria, dysentery and ankylostomiasis. The outbreaks of so-called 'beri-beri,' recorded from time to time amongst British Troops in India and Aden, appear to be capable of explanation in this way.

## II *The effect of adding butter to diets of white flour and 'atta'*

Both white flour and *atta* are deficient in vitamin A, the former more so than the latter. The addition of local butter to the white flour diet, in the proportion of 1.25 gram per rat per day, resulted in an increase of body-weight of from 10.6 to 26.2 per cent, or an improvement of 15.6 per cent. The same amount of local butter when added to the diet of *atta* resulted in an increase in body-weight of from 6.5 to 97.1 per cent, or an improvement of 32.1 per cent. The improvement caused by the butter was thus twice as great when the basis of the diet was whole wheat flour (*atta*) as when it was white flour. It would seem, therefore, that *atta* contained, in greater abundance than white flour, 'something' necessary for the utilization of fats. It must here be added that the amount of fats added to the diets was relatively large.

The results of this experiment are shown in Charts 5 and 6.



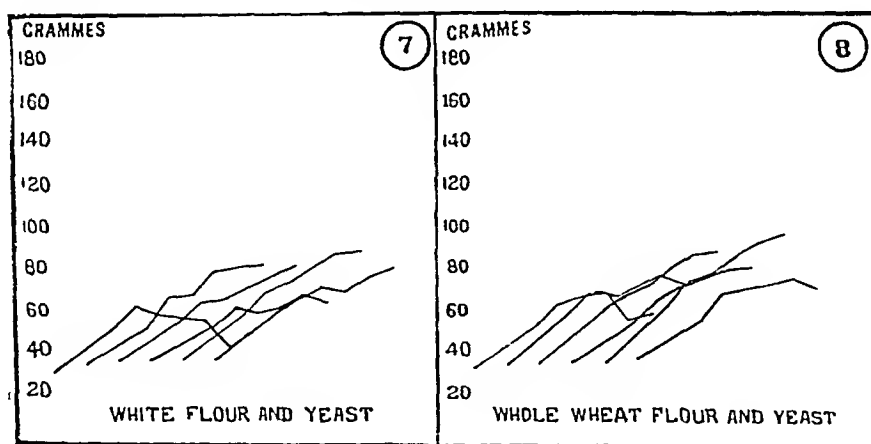


### III The effect of adding 5 per cent of dried yeast to diets of white flour and 'atta'

White flour is deficient in both fractions of vitamin B. The addition of 5 per cent of dried yeast to the white flour diet resulted in an increase of body-weight of 107.9 per cent, or 97.5 per cent more than that resulting from white flour alone, and 42.9 per cent more than that resulting from whole wheat flour alone. It is evident, therefore, that five per cent of dried yeast more than compensated for the deficiency of vitamin B in white flour. In a subsequent experiment (Part II, Experiment I, Series B), it will be shown that 2 per cent of dried yeast does not compensate for the deficiency of this factor in white flour. It may be assumed, therefore, that something between these two figures, say 3.5 per cent of dried yeast, would have sufficed to make the vitamin B-content of the white flour diet equal to that of the whole wheat flour diet.

Whole wheat flour is not deficient in vitamin B<sub>1</sub>, but is relatively poor in vitamin B<sub>2</sub>. The addition of 5 per cent of dried yeast to the *atta* diet caused an increase of body-weight of 121.8 per cent, or an improvement of 56.8 per cent over that resulting from the *atta* diet without the addition of yeast. It may, therefore, be assumed that the yeast provided the vitamin B<sub>2</sub> in which the *atta* was lacking. Yeast is known to exercise some influence on absorption and assimilation(7) favourable to the growth of young rats.

The results of this experiment are shown in Charts 7 and 8.

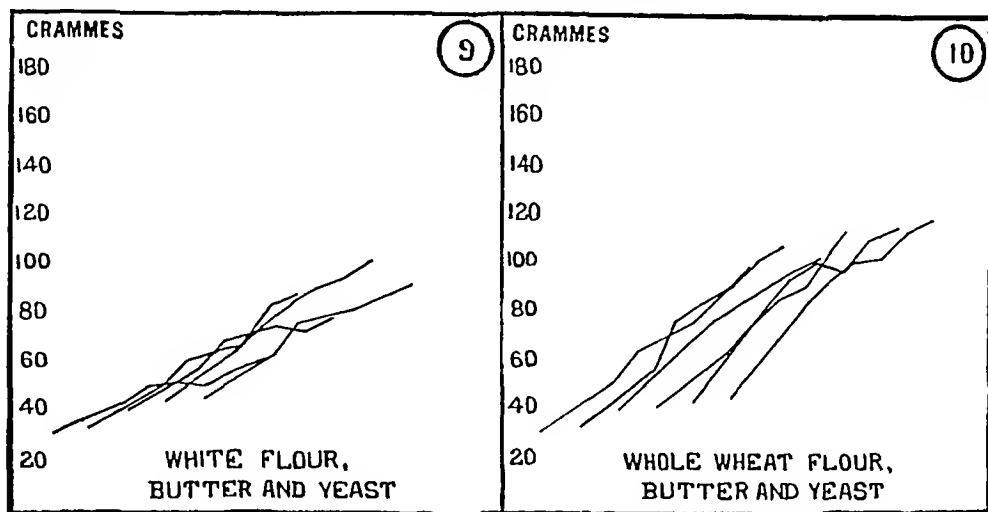


### IV The effects of adding butter and yeast to diets of white flour and 'atta'

The amounts of these substances added to the two diets were of butter 1.25 grams per rat per day, and of yeast 5 per cent. These additions to the white flour diet caused an increase in the rate of growth of from 10.6 to 116.3 per cent, or an improvement of 105.7 per cent. The same addition to the *atta* diet caused an improvement of 112.7 per cent (from 65 to 177.7 per cent). The improvement caused by the addition of butter to the diet of white flour and yeast (*vide* preceding experiment) was trifling (8.4 per cent), while that caused

by the addition of butter to the diet of *atta* and yeast was considerable (55.9 per cent). There was, as previously noted (Experiment II), something in *atta* which assisted in the utilization of fats, this something was deficient in white flour, it appears to be vitamin B.

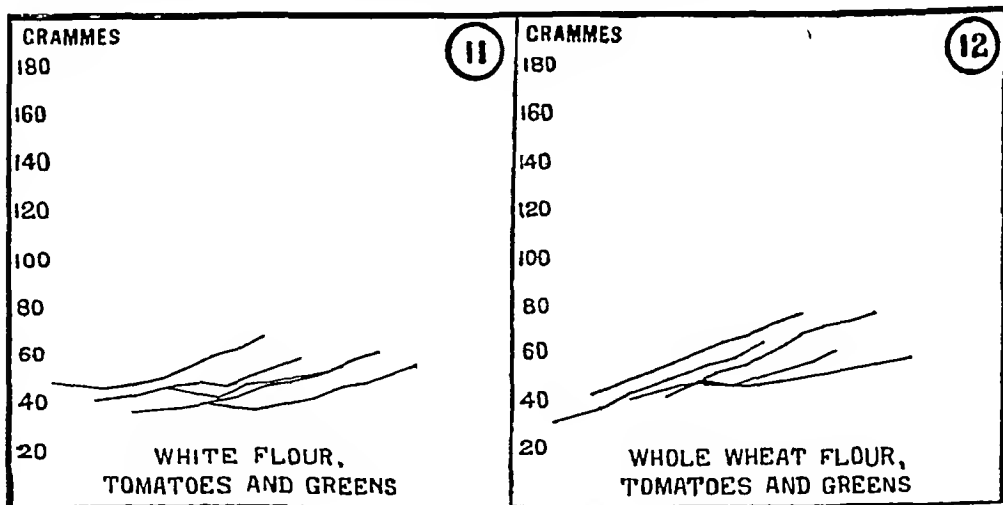
The results of this experiment are shown in Charts 9 and 10.



V *The effect of adding tomatoes and green vegetables to the diets of white flour and 'atta'*

Tomatoes and green leafy vegetables (cabbage), both in the raw state, were given to the animals *ad libitum*. These additions to the white flour diet caused an increase in the rate of growth of from 10.6 to 41.3 per cent, or an improvement of 30.7 per cent. The same additions to the diet of *atta* caused an increase in the rate of growth of from 65 to 68.1 per cent or an improvement of only 3.1 per cent, nevertheless the diet having *atta* as its basis was the better of the two.

The results of this experiment are shown in Charts 11 and 12.

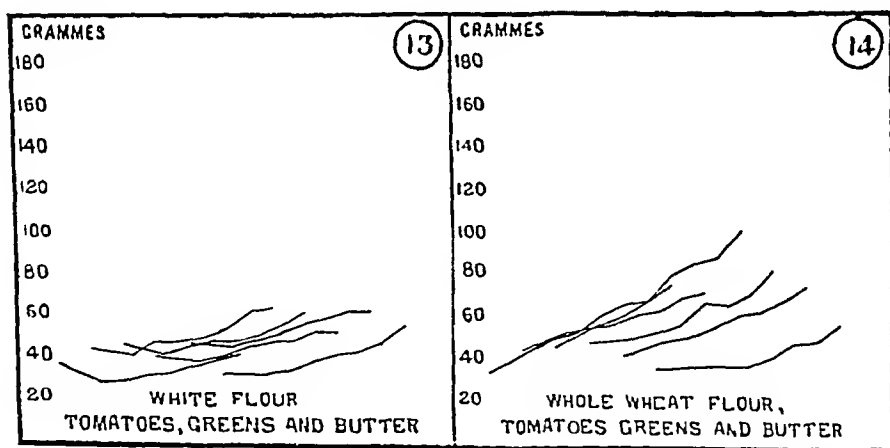


# VI The effect of adding tomatoes, greens and butter to the diets of white flour and 'atta'

The raw vegetables were given *ad libitum*, and the butter in the proportion of 1.25 grammes per rat per day. These additions to the white flour diet caused an increase in the rate of growth of from 10.6 to 35.2 per cent, an improvement of 24.6 per cent. The same additions to the *atta* diet caused an increase in the rate of growth of from 65 to 85.8 per cent, or an improvement of 20.8 per cent.

When the results of this experiment are compared with those of the preceding one, it will be noted that the addition of this amount of butter did not improve the rate of growth resulting from a diet of white flour and vegetables but actually retarded it. The same amount of butter considerably improved the rate of growth resulting from a diet of *atta* and vegetables. This result again points to a deficiency in the white flour diet of something, other than vitamin A, which assists in the utilization of fats: this something is present in whole wheat flour, but is not present to the necessary extent in the tomatoes and greens used, it appears to be vitamin B.

The results of this experiment are shown in Charts 13 and 14.

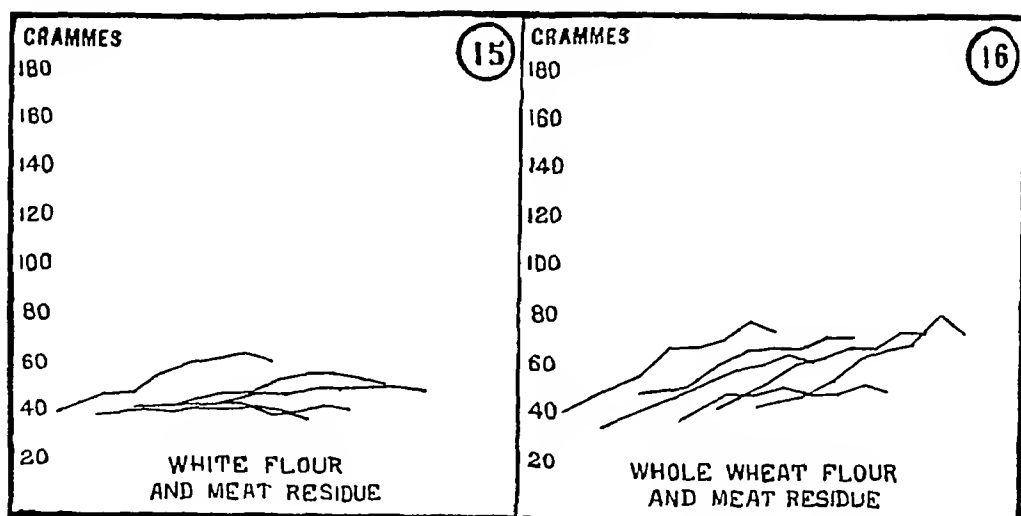


# VII The effect of adding protein to the diets of white flour and 'atta'

In this experiment 10 per cent of the flours was replaced by 'meat residue'\*. The addition of this amount of protein scarcely improved either diet: the white flour diet by 7 per cent and the *atta* diet by 2 per cent.

\*The 'meat residue' was made as follows: lean meat was freed as far as possible from fat, minced, extracted in water, autoclaved in an alkaline medium at 130°C for 1½ hours, dried in the sun and ground to a fine powder.

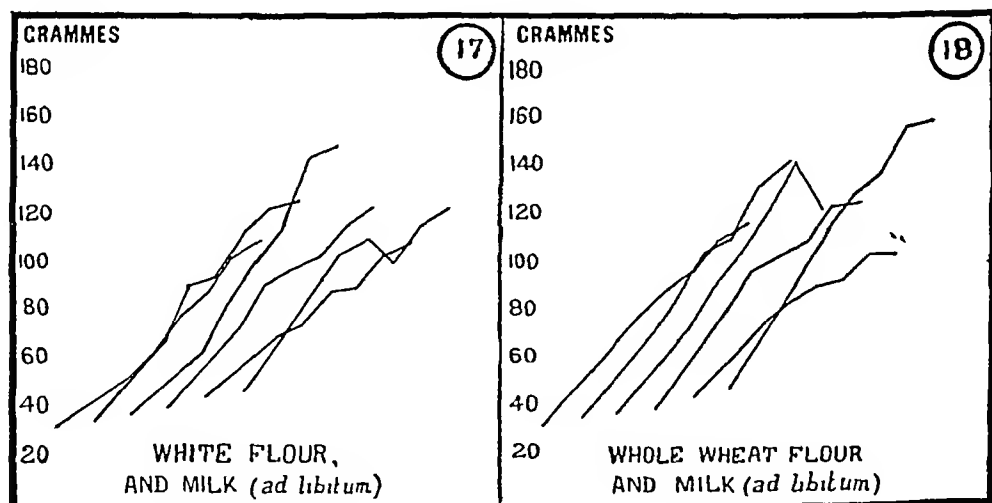
This result is shown in Charts 15 and 16



*VIII The effect of adding whole milk to the diets of white flour and 'atta'*

Whole milk was provided *ad libitum*. The rate of growth on the white flour diet was thereby increased from 10.6 to 209.6 per cent, an improvement of 199 per cent. The rate of growth on the *atta* diet was thereby increased from 65 to 220.4 per cent, or an improvement of 155.4 per cent. The *atta* and milk diet was thus slightly superior to the white flour and milk diet.

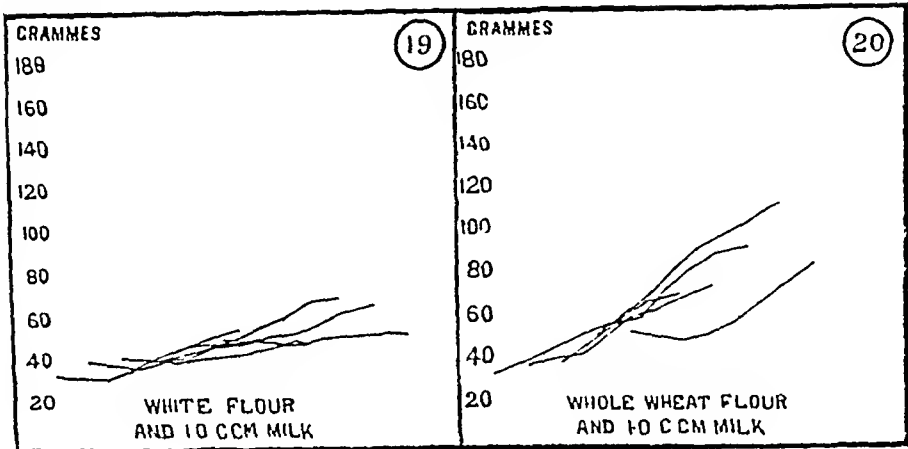
The results are shown in Charts 17 and 18



It was observed that some of the animals in this experiment consumed from 10 to 20 cc of whole milk daily, and that the more milk an animal drank the less it ate of the basal diet. A number of subsidiary experiments under this heading were accordingly carried out, the animals being given measured quantities, ranging from 1 to 10 cc, of whole milk daily. These experiments were continued for 54 days.

(a) The effect of adding 1 c cm of whole milk to the diets of white flour and 'atta'

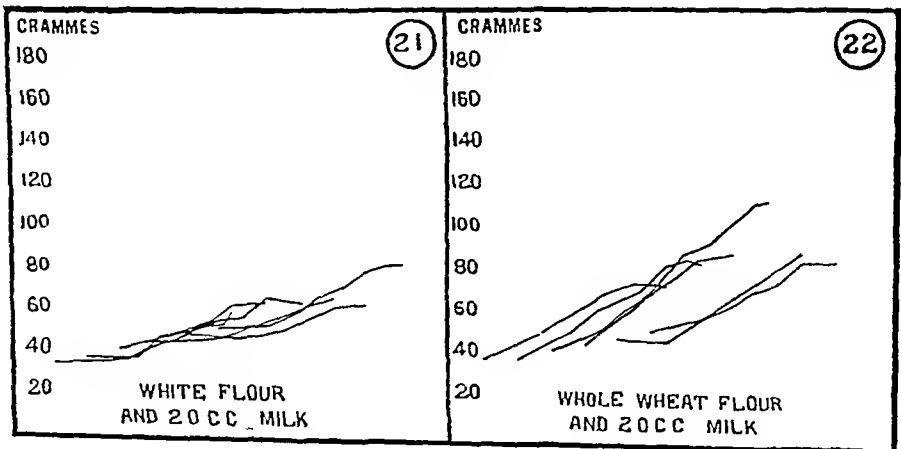
This effect is shown in Charts 19 and 20



The increase in growth on the diet of white flour plus 1 c c of whole milk was 32.2 per cent, on the diet of 'atta' plus 1 c c milk, 81.6 per cent a difference in favour of the *atta* diet of 49.4 per cent

(b) The effect of adding 2 c c of whole milk to the diets of white flour and 'atta'

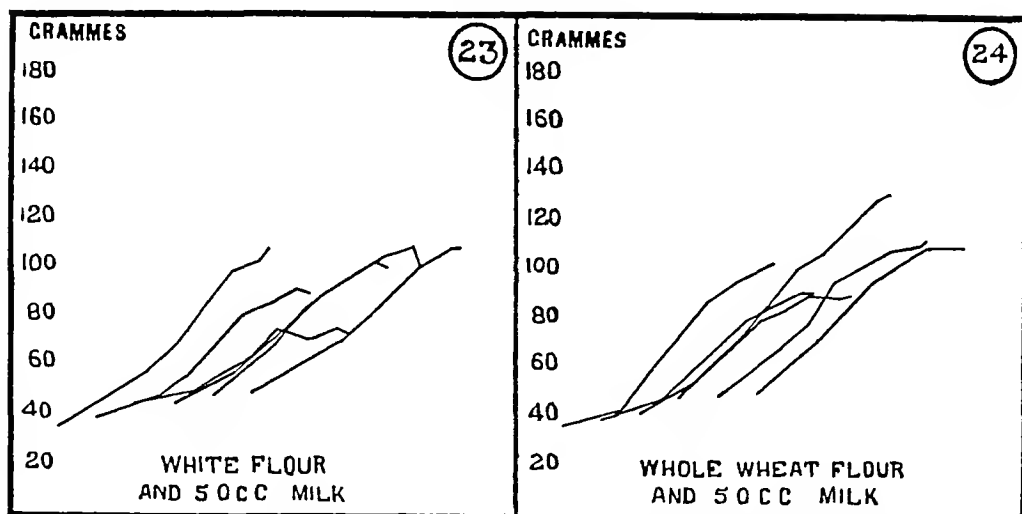
This effect is shown in Charts 21 and 22



The increase in growth on the diet of white flour plus 2 c c of whole milk was 49.4 per cent, on the diet of 'atta' plus 2 c c of milk, 99.9 per cent, a difference in favour of the *atta* diet of 50.5 per cent

(c) The effect of adding 5 c c of whole milk to the diets of white flour and 'atta'

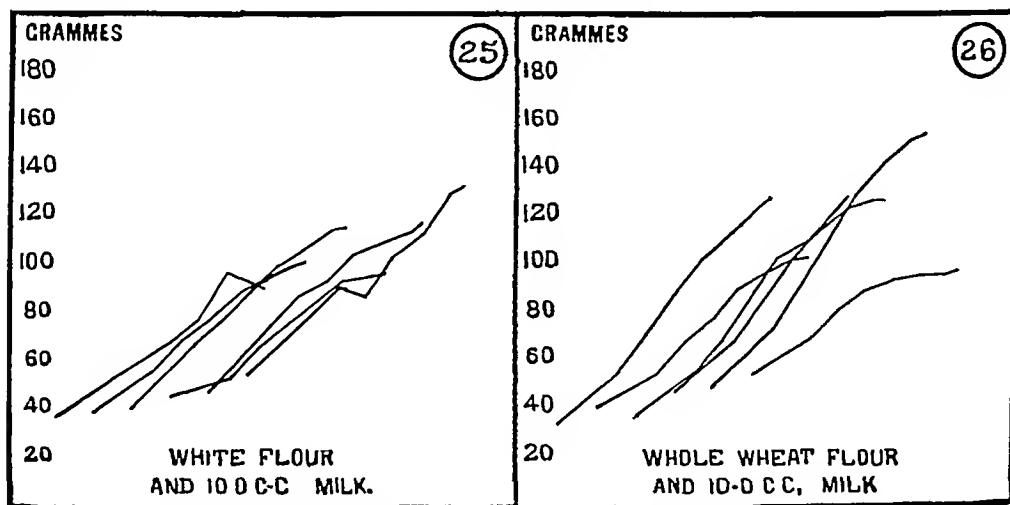
This effect is shown in Charts 23 and 24



The increase in growth brought about by this addition to the white flour diet was 119.5 per cent, and to the *atta* diet, 143.1 per cent a difference in favour of the *atta* diet of 23.6 per cent

(d) *The effect of adding 10 cc of whole milk to the diets of white flour and 'atta'*

This effect is shown in Charts 25 and 26



The increase in growth brought about by this addition to the white flour diet was 149.1 per cent, and to the *atta* diet, 177.2 per cent, a difference in favour of the *atta* diet of 28.1 per cent

The increase in growth of the control rats fed solely on milk, during the same period of 54 days, was 137.3 per cent

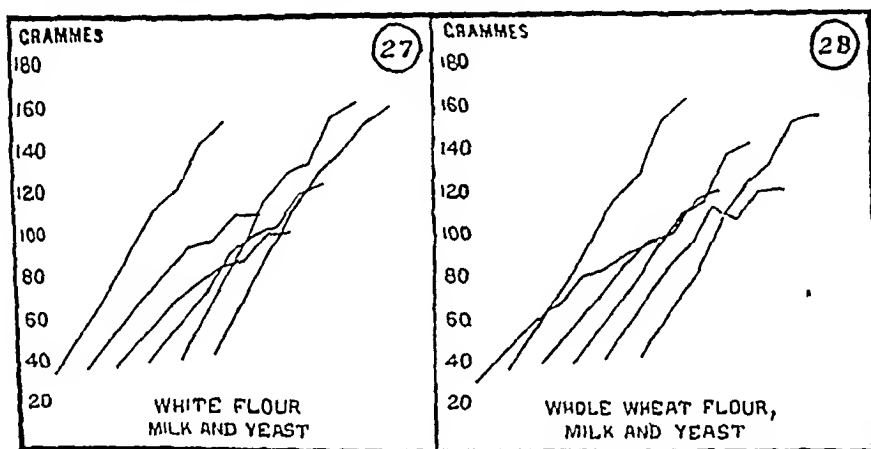
It is thus seen that it is only when whole milk is given *ad libitum* to growing rats, fed on diets of Indian white flour and whole wheat flour (*atta*), that the

growth attained on the former diet tends to equal that attained on the latter. Even then the *atta* maintains a slight superiority over the white flour. This superiority is lost when 5 per cent of dried yeast is added to the diets.

*IX The effect of adding whole milk ad libitum and 5 per cent of dried yeast to the diets of white flour and 'atta'*

These additions to the diets made the one as good as the other, if anything the white flour diet was the better of the two. The increase in body-weight attained with these additions to the white flour diet was 244.6 per cent in 54 days, that attained by the same additions to the *atta* diet was 242.1 per cent in the same period, a difference in favour of the white flour of 2.5 per cent.

This result is shown in Charts 27 and 28.



A summary of the results of these experiments is given in Table II.

TABLE II  
*Summary of results of experiments in Series A*

Experiment	Supplements	AVERAGE PERCENTAGE INCREASE IN BODY-WEIGHT ON THE 54TH DAY OF EXPERIMENT (SPRING MONTHS)	
		White flour	Whole wheat flour
I	<i>Nil</i>	10.6	65.0
II	Butter (125 grammes per rat daily)	26.2	97.1
III	Dried yeast (5 per cent)	107.9	121.8
IV	Butter and dried yeast	116.3	177.7
V	Tomatoes and greens ( <i>ad libitum</i> )	41.3	68.1

TABLE II—*concl'd*

Experiment	Supplements	AVERAGE PERCENTAGE INCREASE IN BODY-WEIGHT ON THE 54TH DAY OF EXPLRIMENT (SPRING MONTHS)	
		White flour	Whole wheat flour
VI	Tomatoes, greens and butter	35.2	85.8
VII	Meat residue (10 per cent)	17.5	67.2
VIII	Whole milk ( <i>ad libitum</i> )	209.6	220.4
	(a) Whole milk (1 cc)	32.2	81.6
	(b) Do (2 cc)	49.4	99.9
	(c) Do (5 cc.)	119.5	143.1
	(d) Do (10 cc)	149.1	177.2
IX	Whole milk ( <i>ad libitum</i> ) and dried yeast	244.6	242.1

The conclusions to be drawn from this series of experiments are as follows —

- (1) Whole wheat flour (*atta*) is superior to white flour as the basal article of diet
- (2) White flour while markedly deficient in vitamin B, contains appreciable amounts of the anti-neuritic fraction ( $B_1$ ) of this vitamin
- (3) The deficiency of vitamin B in white flour is more than compensated for by the addition of 5 per cent of dried yeast. It is estimated that the addition of approximately 3.5 per cent of dried yeast to white flour would suffice to make the vitamin-B-value of the mixture equal to that of whole wheat flour. This is 1.5 per cent more than is used in the manufacture of white bread
- (4) An excess of vitamin B in the form of dried yeast, or some component or components of dried yeast other than vitamin B, is favourable to the growth of young rats fed on a basal diet of either white flour or whole wheat flour
- (5) There appears to be something in whole wheat flour which assists in the utilization of fats, this something is deficient in white flour. It is probably vitamin B, acting in association with vitamin A
- (6) The addition of tomatoes and green leafy vegetables (*ad libitum*) to diets of white flour or of whole wheat flour does not make good their deficiencies, though it improves the nutritive value of the diets
- (7) The addition of 10 per cent of protein to diets of white flour and of whole wheat flour improves their nutritive value but little



- (8) The addition of whole milk to diets of white flour and of whole wheat flour improves their nutritive value proportionately to the amount of milk added
- (9) When whole milk is supplied *ad libitum* to young rats, together with 5 per cent of dried yeast, it does not matter whether the basis of the diet be white flour or whole wheat flour. But in these circumstances the main constituent of the diet is milk, and it is consumed by the rats in amounts out of all proportion to those available for the average human being under modern conditions of life. Even when whole milk is consumed in considerable quantities (10 c.c. per rat per day), whole wheat flour maintains its superiority over white flour as the staple article of the diet

## PART II.

### White bread *versus* whole wheat unleavened bread 'chapatti'

The white bread used in this part of the investigation was obtained from the local bakery. It contains approximately 2 per cent of yeast and is made from Indian white flour. The unleavened bread (*chapatti*) was made from locally grown wheat (i.e., wheat grown in the Nilgiri Hills, where Coonoor is situated)

[This wheat is of poor quality. The soil on which it is grown is poor. The crop matures rapidly, sowing takes place early in April, harvesting late in June.]

The wheat was freshly ground in the laboratory every day. After removal of the coarser particles of bran, by sifting, the resultant whole wheat flour was made up into a dough with water and rolled out into thin cakes which were lightly cooked, on an iron plate over an open fire, in the way customary in India. The *chapattis*, so made, contained neither yeast nor salt.

This series of experiments was carried out during the autumn months, the previous series having been carried out in the spring. The same experimental procedure was followed as in the previous series and the same precautions were taken as to the choice of animals and the sex-composition of each group. The experimental period was the same in both series—54 days. The results are not strictly comparable, since the whole wheat flour used in the first series was of a different quality to that used in the second, and young rats grow best in the spring months in this locality.

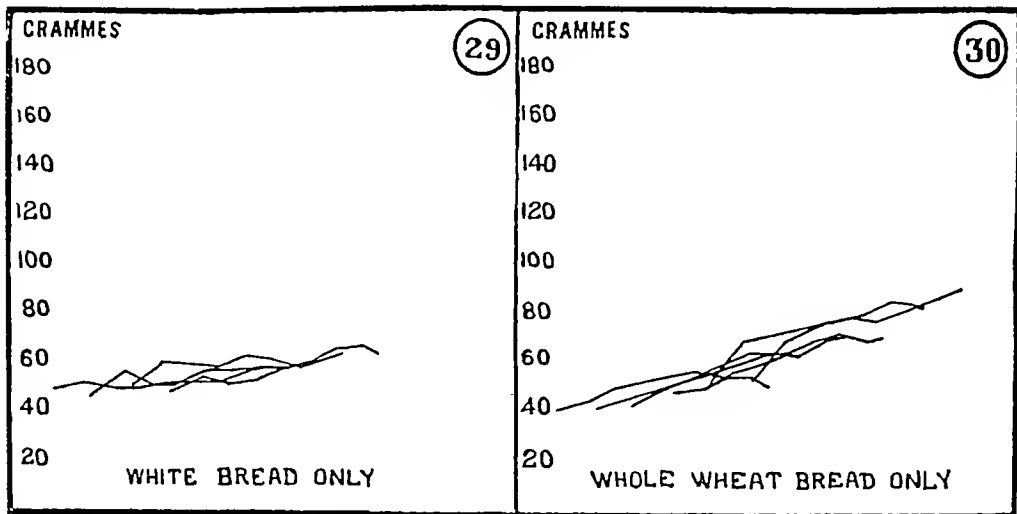
### Series B

#### I The effect on growth of exclusive diets of white bread and 'chapattis'

The results of this experiment are shown in Charts 29 and 30.

The percentage increase in body-weight of young rats fed on the exclusive diet of white bread was 21.2, and of those fed on *chapattis*, 53.7—a difference in favour of the whole wheat *chapattis* of 32.5 per cent. The 2 per cent of yeast

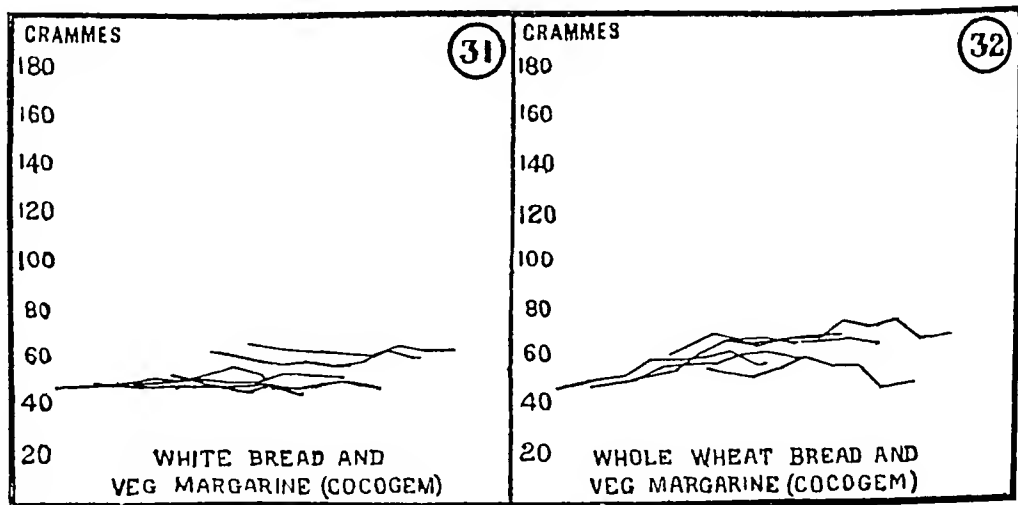
used in the making of white bread does not make the vitamin B-content of the bread equal to that of whole wheat unleavened bread (*chapatti*)



## II *The effect of adding vegetable margarine to diets of white bread and 'chapattis'*

The vegetable margarine was cocogem a hardened fat, made from coconut oil, widely used in India. It was added to the two diets in the proportion of 1.25 grammes per rat per day.

The results are shown in Charts 31 and 32.



The addition of this amount of cocogem to the diet of white bread inhibited the growth of young rats and caused an *actual loss of weight* amounting on the average to 2.4 per cent by the 54th day. This loss of weight, compared with the retardation of growth induced by the same amount of butter (*vide infra*), appears to indicate not only that the intake of fat was excessive and harmful,

but that the degree of harmfulness was dependent to some extent on the degree of deficiency of vitamin A in the fat

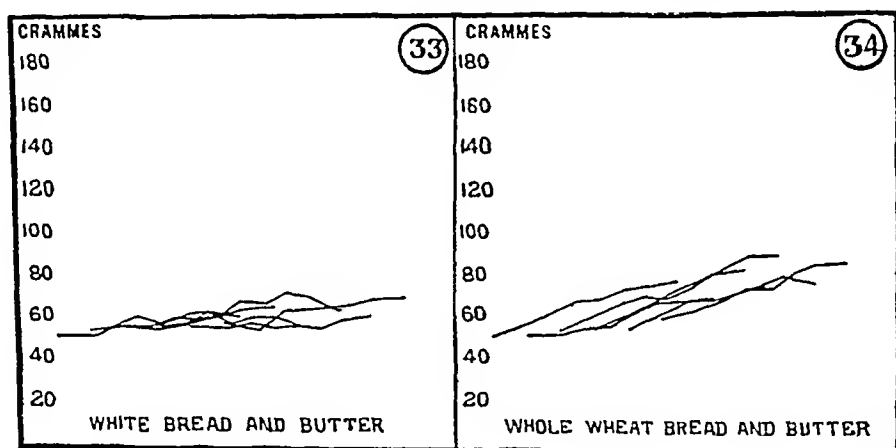
The addition of the same amount of cocogen to the diet of *chapattis* also resulted in a retardation of growth the percentage increase in body-weight being only 12.7 as compared with 53.7 per cent when the animals were fed on *chapattis* alone

The use of this vitamin A poor fat in considerable amounts either with white bread or with whole-wheat, unleavened bread (*chapatti*) is definitely harmful, a diet of white bread and vegetable margarine (cocogen) being particularly so

### III The effect of adding butter to the diets of white bread and 'chapattis'

The amount of butter added was 1.25 grammes per rat per day a fairly high proportion of the total food-intake

The results of the experiment are shown in Charts 33 and 34



The addition of this amount of butter to the diet of white bread caused an increase in body-weight of only 15.4 per cent by the 54th day or a drop in weight of 5.8 per cent when compared with the weight of rats fed on the white bread alone. The butter was actually harmful.

The same amount of butter when added to the diet of *chapattis* caused an increase in body-weight of 51.7 per cent by the 54th day, or 2 per cent less than that of rats fed on *chapattis* alone. Here also the addition of butter was harmful, but much less so than when the basal diet consisted of white bread.

Clearly, the young animals were not able to deal with this amount of fats, but their ability to do so was markedly less when the basal diet was white bread than when it was whole wheat, unleavened bread.

The rate of growth of the animals in this experiment compared unfavourably with that of animals in a similar experiment (II) in Series A. Three factors

may have been concerned in this result seasonal influences on growth, the poorer quality of the wheat flour, and a lower content of vitamin A in the butter used in this series

Unfortunately, the experiments did not include one in which dried yeast was added to the diets of white bread and of whole wheat bread at the same time as the butter or cocogem. But from the results of the experiments in the previous series, in which 5 per cent of dried yeast was added to the diets of white flour and butter and of *atta* and butter, it may be concluded that an abundant provision of vitamin B is, in addition to vitamin A, necessary for the metabolism of the amount of fats added to the diets in these experiments (*vide* previous Series II, III, IV and VI). It was not only the lack of vitamin A in the vegetable margarine and its insufficiency in the butter but the deficiency of vitamin B in the white flour and its insufficiency in the low grade, whole wheat flour which was responsible for the harmful effects of butter and cocogem in the amounts used in these experiments. Even in the better (Punjab) whole wheat flour there was not enough vitamin B to compensate for 1.25 grammes of fat in the food, for the addition of 5 per cent of dried yeast to a diet of this flour and butter caused the body-weight to be nearly doubled in 54 days (*cf* Charts 6 and 10), thus indicating that the vitamin B provided by the yeast aided in the utilization of the fats.

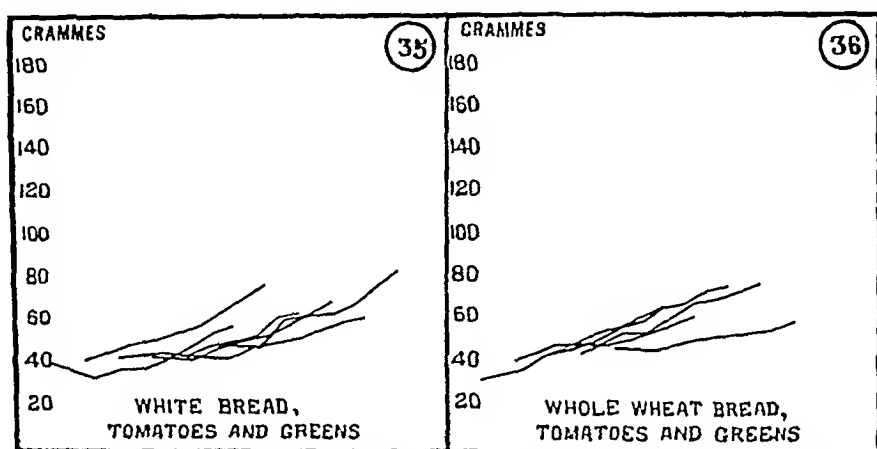
There is at the present time considerable controversy as to the supposed deleterious effects of an excess of vitamin A in the food, it having been observed by Hopkins(8) that an excess of cod-liver oil is harmful to young rats, as indeed, it is harmful to young children. The results of the experiments here reported appear to indicate that this deleterious effect is due less to an excess of vitamin A than to an excess of fats. The observation made by Hopkins(8) that the retardation of growth in rats brought about by an excess of cod-liver oil could be prevented by administering an excess of vitamin B is possibly to be explained not so much by the necessity for a proper balance of vitamins A and B in the diet—though no doubt this is of importance—as by the necessity for an abundance of both vitamin A and vitamin B for the metabolism of fats.

Some misgiving exists in India as to the greatly increased use of 'vegetable ghees' as articles of diet, it may be well, therefore, to state what the merits and demerits of these 'ghees' are. Vegetable ghees have a certain culinary and fuel value while they possess, when sold in sealed tins, the great advantage of being clean, but their lack of vitamin A is a great disadvantage to their use, and for this reason alone they must be regarded as harmful when they provide the major part of the fats consumed. When they are used in considerable quantities in a diet deficient in vitamin B, their effects may be still more serious. The contemporaneous increase in the use of white bread and 'vegetable ghees' is a grave departure from customary usage that cannot fail to have deleterious effects on public health.

#### IV The effect of adding tomatoes and greens to the diets of white bread and 'chapatti'

These vegetables were provided *ad libitum* the greens being given in the form of fresh cabbage leaves

The results of the experiments are shown in Charts 35 and 36



The addition of raw tomatoes and greens to the white bread diet caused an increase in body-weight of 35.1 per cent over that resulting from white bread alone (56.3 per cent as compared with 21.2 per cent). The same additions to the *chapatti* diet caused an increase in body-weight of from 53.7 to 68.1 per cent, an improvement of 14.4 per cent. The results of this experiment indicate the value of an abundant supply of vegetables especially in diets of which the basis is white bread.

#### V The effect of adding tomatoes, greens and vegetable margarine to the diets of white bread and 'chapatti'

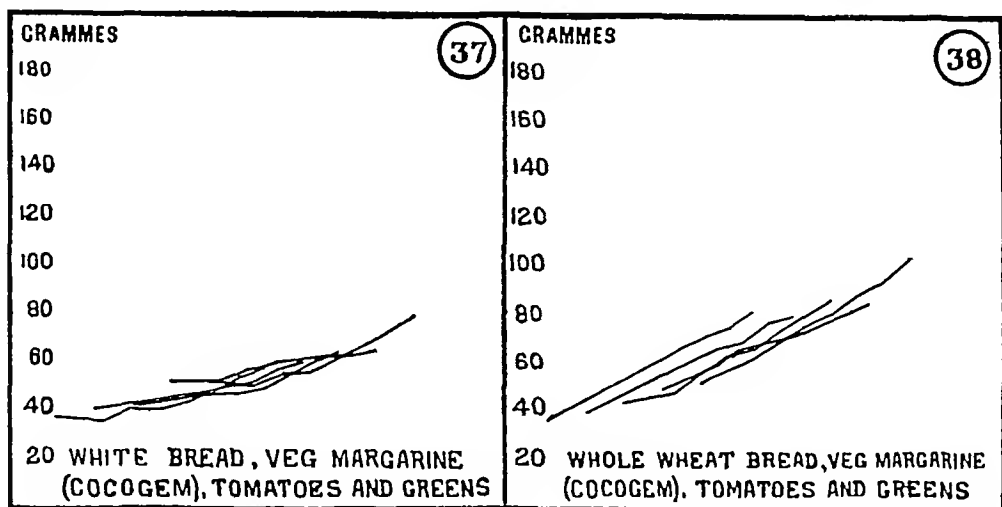
The vegetables were provided *ad libitum*, the greens being given as cabbage leaves. The vegetable margarine was given in the form of 'cocogem' 1.25 grammes per rat per day.

The results are shown in Charts 37 and 38.

These additions to the diet of white bread caused an increase of body-weight amounting to 47.2 per cent after 54 days, an improvement of 26 per cent over the weight resulting in the same period from the diet of white bread alone, a still more marked improvement over that resulting from a diet of white bread and cocogem, but a retardation of growth as compared with that resulting from a diet of white bread and vegetables without added fats. Here again the deleterious effects of the vitamin-A-free fats, in the absence of a sufficiency of vitamin B, are apparent.

The same addition to the *chapatti* diet caused an increase in body-weight amounting to 94.5 per cent, an improvement of 81.8 per cent over that resulting

from a diet of *chapattis* and cocogem, and of 26.4 per cent over that resulting from a diet of *chapattis* and vegetables. It is evident, therefore, that the



tomatoes and greens provided 'something' which assisted in the utilization of the vegetable fats added to the diet. From what has preceded it appears reasonable to conclude that this 'something' was vitamin B acting in association with the vitamin A contained in the greens and tomatoes. The quantity of these factors provided by the vegetables was not adequate when the basis of the diet was white bread, it was more adequate when the basis of the diet was whole wheat, unleavened bread.

#### VI *The effect of adding tomatoes, greens and butter to the diets of white bread and 'chapattis'*

The vegetables were provided *ad libitum*, the greens being given as cabbage leaves. The butter was given in the proportion of 1.25 grammes per rat per day.

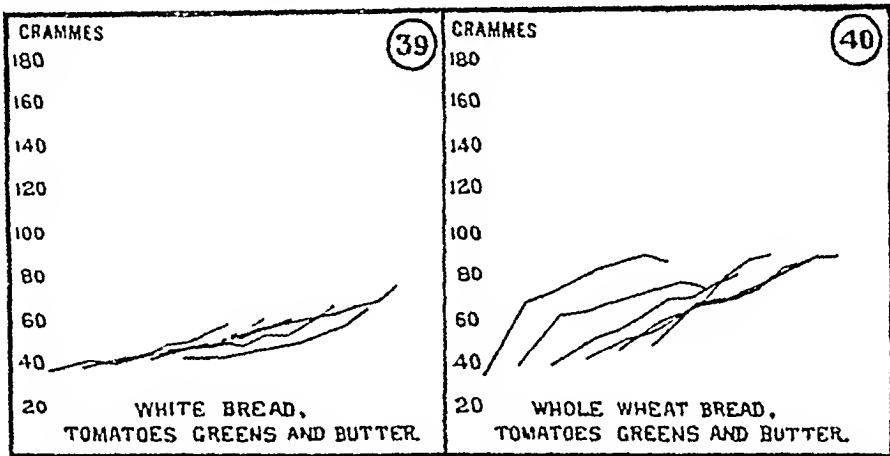
The results are shown in Charts 39 and 40.

These additions to the diet of white bread caused an increase of body-weight amounting to 51.7 per cent after 54 days, an improvement of 30.5 per cent over the weight resulting in the same period from the diet of white bread alone, and of 36.3 per cent over that resulting from a diet of white bread and butter, but a retardation of growth, amounting to 4.6 per cent, when compared with the growth resulting from a diet of white bread, tomatoes and greens without butter.

The same additions to the diet of *chapattis* caused an increase of body-weight amounting to 97.3 per cent after 54 days, an improvement of 43.6 per cent over the weight resulting in the same period from the diet of *chapattis* alone, of 45.6 per cent over that resulting from a diet of *chapattis* and butter, and of 29.2 per cent over that resulting from a diet of *chapattis*, tomatoes and greens.

The results of this experiment indicate again that an abundant supply of vitamin B is necessary for the utilization of the relatively large amount of butter fat added to the two diets. A diet of white bread and vegetables *ad libitum*

did not ensure a sufficient supply of this factor, where as a diet of whole wheat bread (*chapatti*) and vegetables *ad libitum* ensured a more adequate supply of

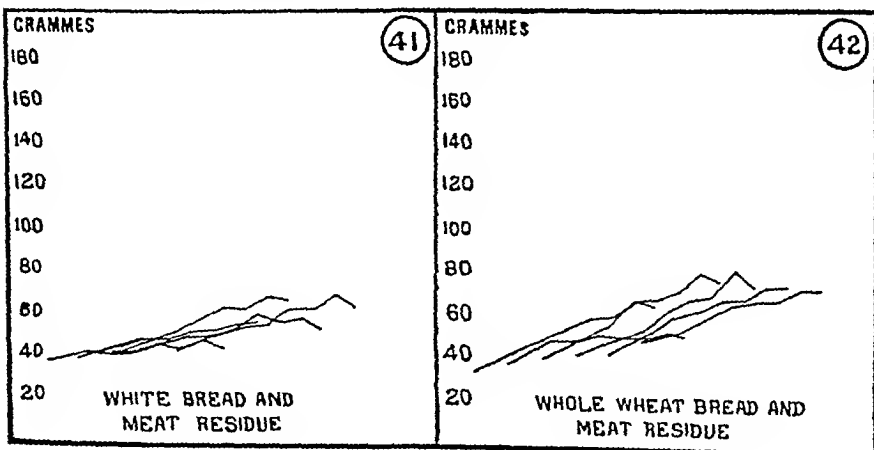


it owing to the higher content of vitamin B in the latter bread. Compared with the results of the previous experiment in which the same amount of a vegetable margarine, containing no vitamin A, was used, the results indicate the beneficial effects on growth of the vitamin A contained in the butter, though the comparatively slight differences in the results of the two experiments would appear to show that the butter was relatively poor in vitamin A.

VII The effect of adding 10 per cent of protein to the diets of white bread and 'chapattis'

The protein was added in the form of 'meat residue' (*vide ante*). An addition of 10 per cent improved the rate of growth resulting from white bread alone by 17.9 per cent, and that resulting from *chapattis* alone by 13.2 per cent.

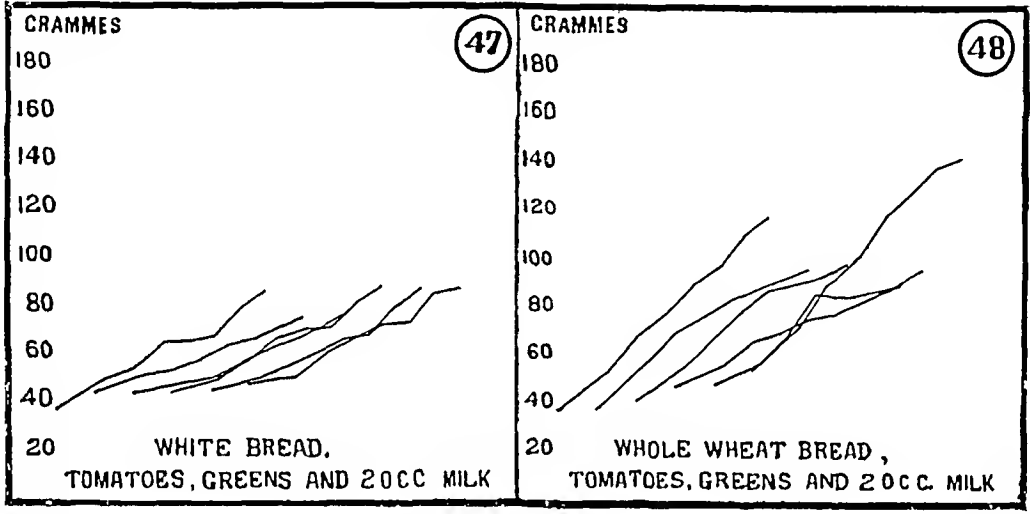
These results are shown in Charts 41 and 42.



*X The effect of adding tomatoes, greens and milk to the diet of white bread and 'chapattis'*

The tomatoes and greens were provided *ad libitum*, the milk in the proportion of 20 c.c. per rat per day

These additions to the white bread diet caused an increase of body-weight which amounted to 92.9 per cent in 54 days, and to 139.6 per cent in the same period when made to the diet of *chapattis*. The results are shown in Charts 47 and 48



A summary of the results of these experiments is given in Table III

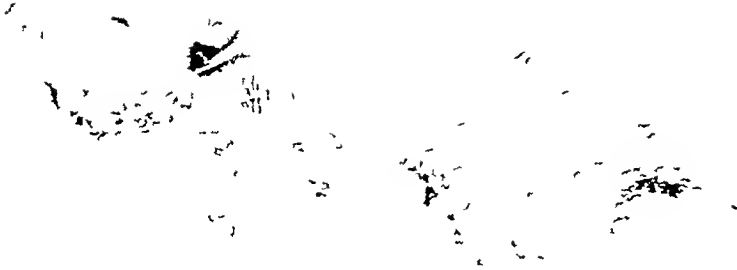
TABLE III  
*Summary of results of experiments in Series B*

Experiment	Supplements	AVERAGE PERCENTAGE INCREASE OR DECREASE OF BODY-WEIGHT ON THE 54TH DAY OF THE EXPERIMENT (AUTUMN MONTHS)	
		White bread	Whole wheat chapattis
I	Nil	+21.2	+53.7
II	Vegetable margarine (cocogem) (1.25 grammes)	-2.4	+12.7
III	Butter (1.25 grammes)	+15.4	+51.7
IV	Tomatoes and greens ( <i>ad libitum</i> )	+56.3	+68.1
V	Tomatoes, greens and vegetable margarine	+47.2	+94.5
VI	Tomatoes, greens and butter	+51.7	+97.3
VII	Meat residue (10 per cent)	+39.1	+66.9
VIII	Whole milk (2.5 c.c.)	+71.2	+80.5
IX	Whole milk (2.5 c.c.), vegetable margarine (1.25 grammes)	+49.2	+102.1
X	Tomatoes, greens and milk (2 c.c.)	+92.9	+139.6





PLATE L



1—Rat No 431 fed on a diet consisting of white flour 60 parts, meat residue 20 parts, olive oil 10 parts, and McCollum's salt mixture 5 parts. Paralysis appeared in the hind limbs the 75th day thereafter spreading to the forelimbs. Death occurred on the 86th day. Weight curve shown in Chart 3.



Fig 2—Rat No 1140 fed on a diet consisting of white flour 77 parts, meat residue 10 parts, olive oil 8 parts, and McCollum's salt mixture 5 parts. Paralysis appeared on the 156th day. Death occurred on the 164th day. Weight curve shown in Chart 4.

The conclusions to be drawn from this series of experiments are —

- (1) White bread, containing approximately 2 per cent of yeast, is inferior in nutritive value to unleavened bread (*chapatti*) made from whole wheat flour
- (2) The amount of yeast used in the manufacture of white bread does not suffice to make good the deficiency of vitamin B in the white flour from which the bread is made
- (3) The superiority of whole wheat bread (*chapatti*) over white bread as the staple article of diet is maintained even when the diet is supplemented with an abundance of fresh vegetable foods and 2 c.c. of whole milk—an amount of milk which would correspond to over a pint a day for a child of 7 years old
- (4) When fats form a considerable proportion of the diet, an abundant supply of vitamin B, as well as of vitamin A, is necessary for their utilization. A diet having white bread as its basis does not provide these factors in amounts sufficient for this purpose even when it is supplemented with a fair amount of whole milk and an abundance of fresh vegetable foods, on the other hand, a similarly constituted diet having whole wheat bread as its basis, does provide these vitamins in sufficient amounts
- (5) Amongst the properties possessed by milk which render it unsurpassed in excellence as a dietary constituent for growing rats—or children—is its ability to aid in the utilization of fats

These results, together with those arrived at from a comparative study of the two flours (page 680), leave no room for doubt that whole wheat, unleavened bread (*chapattis*) is markedly superior to white bread as the basal article of diet. Both breads must, however, be adequately supplemented with the foods so rightly designated 'protective' by McCollum—fresh vegetables and whole milk. The effects of an insufficiency of these 'protective foods' in the dietary cannot properly be held to detract from the great value of whole wheat bread as a basal article of diet.

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# THE EFFECT ON THE TRACHEAL MUCOUS MEMBRANE AND ON THE THYROID GLAND OF FAULTY FOOD CONTAINING AN INSUFFICIENCY OF VITAMIN A

BY

BREAST-COLONEL R McCARRISON, C I R, K I P, M D, F R C P, I M S,  
*Director, Nutritional Research, I R F A, Pasteur Institute, Coonoor, S India*

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Stock rats in this laboratory receive a diet consisting of whole wheat flour (*chapattis*), sprouted dhal (*pulse*), butter, green vegetables *ad libitum* and a liberal supply of whole milk. Twice a month they are given a small ration of raw meat. On this diet they remain in perfect health, are very prolific and the death-rate is very low. When it is desired to reduce the birth-rate, butter and meat are excluded from the dietary and the amount of fresh vegetable food and milk is reduced to a minimum. Some time ago it became necessary to reduce the birth-rate amongst the stock animals, accordingly, a number of them were fed, for a period of 12 months, on a diet consisting of a whole wheat *chapattis*, dhal, and a small daily ration of green vegetables and diluted milk. The chief fault of this restricted diet is its low content of vitamin A, it is also deficient in vitamin C and D, and in certain mineral elements. Its iodine-content was not determined, but as the components of the diet were local products, derived from an iodine-rich soil,\* it may be assumed that iodine was not lacking in them. The manganese-content of the diet was relatively high.

It occurred to me to examine the thyroid glands of a number of the animals fed on this diet, with the object of determining whether or not prolonged insufficiency of vitamin A, in association with the other food-faults above mentioned, had any effect on this organ. For this purpose 23 rats, aged between 18 and 24 months, were killed and the trachea with the attached thyroid gland removed from each. No anterior nor lateral enlargement of the thyroid gland was noted,

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\* Coonoor is situated at a height of 6,000 feet above sea-level. Its soil and that of the surrounding country is rich in iodine. Endemic goitre is unknown, though 'thyroid swellings' (mostly physiological) were found in 24 per cent of girls in a local school.

but in some animals its lobes were observed to meet behind the trachea. The tracheæ with the attached thyroid glands were serially sectioned, each section being 8  $\mu$  thick. An examination of these sections revealed, in a proportion of the cases, three abnormal features

- (a) Proliferative changes of varying degrees of intensity in the tracheal mucous membrane of 15 of the 23 animals
- (b) Abnormal distension of the follicles of the thyroid gland in 11 of the 15 cases showing proliferative changes in the tracheal mucous membrane
- (c) A backward growth of the posterior borders of the lateral lobes of the thyroid gland so that they met behind the trachea. This change was observed in 5 of the 23 cases, though not always in those presenting the most marked degrees of proliferative change in the tracheal mucous membrane

It may here be added that none of these changes have been observed in well-fed stock rats, and that endemic goitre does not occur amongst them

#### **Proliferative Changes in the Tracheal Mucous Membrane.**

Several observers, notably Wolbech and Howe(1 and 2) have drawn attention to the metaplastic changes occurring in the mucous membrane of the respiratory tract as a result of *deprivation* of vitamin A. The changes consist in a transformation of the columnar, ciliated epithelium lining the trachea and bronchi into stratified, keratinizing epithelium. This type of metaplasia is characteristic of vitamin A deficiency. It is not peculiar to the epithelium of the respiratory tract but may involve epithelial structures throughout the body. In this laboratory it has frequently been observed(3) in rats fed on diets composed mainly of cereal products—oatmeal, whole wheat flour and white flour—and having deficiency of vitamin A as one of their chief faults. In the respiratory passages this type of metaplasia may occur alone or in association with proliferative changes in the sub-epithelial tissue of the mucous membrane(3). In the animals with which the present paper deals keratinization of the tracheal epithelium was occasionally observed, but proliferative changes of greater or lesser degrees of intensity dominated the histological picture.

The proliferative process—illustrated in Plate LI, fig. 1 and Plates LII and LIII, figs. 3 to 10—appeared to be the result of a chronic and protracted inflammatory reaction in which mononuclear cells, in greater or lesser numbers, pervaded the sub-epithelial layer of the mucosa, polynuclear cells being comparatively rare. Moderate engorgement of the mucosal vessels was present in some cases but was inconspicuous in others, while the glandular elements of the mucous membrane showed varying degrees of degenerative change. In a few cases, especially those in which polypoid projections were marked, distinct evidence of inflammation was so inconspicuous that some doubt was felt as to whether the condition was not one of hyperplasia of the lymphoid elements of the mucous membrane.

The process varied in degree and in the extent to which the mucosa was affected by it. Sometimes it was quite local, and confined to one quadrant of the trachea, or formed a small nodule which projected slightly into the tracheal lumen (Plate LII, figs 3 and 4). As a rule the tracheal mucous membrane was involved in its whole circumference, though not necessarily in its whole longitudinal extent. In some cases the thickening of the mucosa, to which the process gave rise, was relatively slight (Plate LII, fig 5), while in others the mucous membrane was thrown into polypoid (Plate LIII, fig 9) or villiform (Plate LIII, fig 10) elevations which greatly reduced the lumen of the trachea. Between these extremes all grades of proliferative change and resultant thickening of the mucous membrane were met with (Plate LII, fig 6 and Plate LIII, figs 7 and 8).

The thickened mucosa was either wholly or partially denuded of covering epithelium, or was covered in a patchy way with one or more layers of keratinizing cells. Occasionally, in clefts between villiform (Plate LIII, fig 10) or polypoid (Plate LIII, fig 9) projections, the columnar character of the epithelium was preserved though its cells were degenerated.

In looking at some of these polypoid masses,—which might almost be called ‘tracheal adenoids’—one is forcibly reminded of the chronic tonsillar hypertrophy and hyperplasia of the naso-pharyngeal adenoid tissues (‘adenoids’) so frequently found in badly nourished children. A process such as that here described, were it to occur in the upper reaches of the respiratory tract, might well give rise to, or assist in the production of, these conditions in children. It is reasonable, therefore, to expect that prolonged subsistence on faulty diets of low vitamin-A-content may be a factor in their causation.

### Changes in the Thyroid Gland

These changes consisted in distension of a large proportion of the thyroid follicles with colloid material and frequent distortion of their shape, secretory activity being present in parts of the gland not affected by follicular distension.

The normal thyroid gland of healthy, well-fed rats living at this altitude exhibits a certain uniformity as to the size and shape of its follicles. These may vary to some extent in different areas of the same resting gland, often being larger in its peripheral than in its more central parts. But there is a limit, both as to their size and shape, which is not passed under normal conditions of nutrition and health. Such distension and distortion of the thyroid follicles as were met with in a proportion of the rats in this series are definitely abnormal. The character of this change is best appreciated by a reference to the accompanying figures. Plate LIV, figs 11 and 12 are sections through the thyroid and trachea at the level of the isthmus of the gland: the one (Fig 11) shows the normal appearances of the thyroid follicles and of the tracheal mucous membrane, the other (Fig 12)

shows the abnormally distended follicles and the proliferative changes in the tracheal mucosa. Similar changes were present in the main body of the gland (Plate LV, figs 14 to 17). But while distension, and sometimes distortion of the follicles, was the most conspicuous feature of the abnormal thyroids, the glandular tissue was not given up wholly to colloid storage. Thus, in Figs 14 and 15 a number of small vesicles are to be seen in which active secretion is proceeding side by side with colloid storage in the larger follicles. Both phases of the thyroid gland's activity were thus in evidence, though colloid storage was the more conspicuous. So conspicuous, indeed, was the latter phase in some cases (Plate LI, fig 2 and Plate LV, figs 16 and 17) that, had the glands exhibiting it been enlarged, a diagnosis of 'colloid goitre' would have been justifiable.

It is remarkable that the thyroids presenting these changes were not always enlarged. In a few cases there was a backward growth of the posterior borders of the lateral lobes so marked as to cause the trachea to be completely enveloped by thyroid tissue (Plate LIV, fig 13). This envelopment is distinctly abnormal and indicates that the changes occurring in the thyroid did give rise some animals to thyroid enlargement.

The proliferative changes in the tracheal mucous membrane, when of marked degree, were associated so consistently with distension of the thyroid follicles with colloid material as to suggest a cause and effect relationship. It cannot, of course, be concluded as a result of examining relatively small numbers that the degree of follicular distension in the thyroid was dependent on the gravity of the proliferative changes in the trachea. Nevertheless, there is some outside evidence that this may have been so. Blauel and Reich(4) have shown that if the trachea of a normal dog be constricted, by the application of a silk ligature, the follicles of the thyroid became distended with colloid. This observation, which appears to have been confirmed by Breitner,(5) may have a bearing on the follicular distension observed in the thyroid glands of rats, the lumen of whose trachea was narrowed by chronic proliferative changes consequent on faulty food containing an insufficiency of vitamin A. Breitner(5) has recorded that the accumulation of colloid in the dog's thyroid, which results from surgical stricture of the trachea, can be prevented by the administration of iodine. Whether iodine would have had the same effect on the thyroid glands of rats suffering from dietetically-induced stricture of the trachea is an interesting question.

The purpose of this paper has been to record certain observations with regard to the effects of insufficiency of vitamin A, in association with certain other food-faults, on the thyroid gland and on the mucous membrane of the respiratory tract. But it is evident that these observations may be pertinent to other matters requiring elucidation. Amongst these are the possible relation of faulty food containing an insufficiency of vitamin A to chronic hypertrophy of the tonsils and to hyperplasia of the naso-pharyngeal adenoid tissues, and the possible relation of insufficiency of vitamin A to iodine metabolism and to colloid goitre-formation.



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#### EXPLANATION OF PLATE L.I

- Fig 1 Section of trachea of rat showing normal appearance of mucous membrane low power magnification
- „ 2 Section of trachea and thyroid of rat showing great thickening of the tracheal mucous membrane with polypoid projections into the lumen of the wind-pipe, suggesting adenoid growths Note the distension and distortion of the thyroid follicles suggesting 'colloid goitre' formation The thyroid gland was not, however, obviously enlarged magnification as in Fig 1

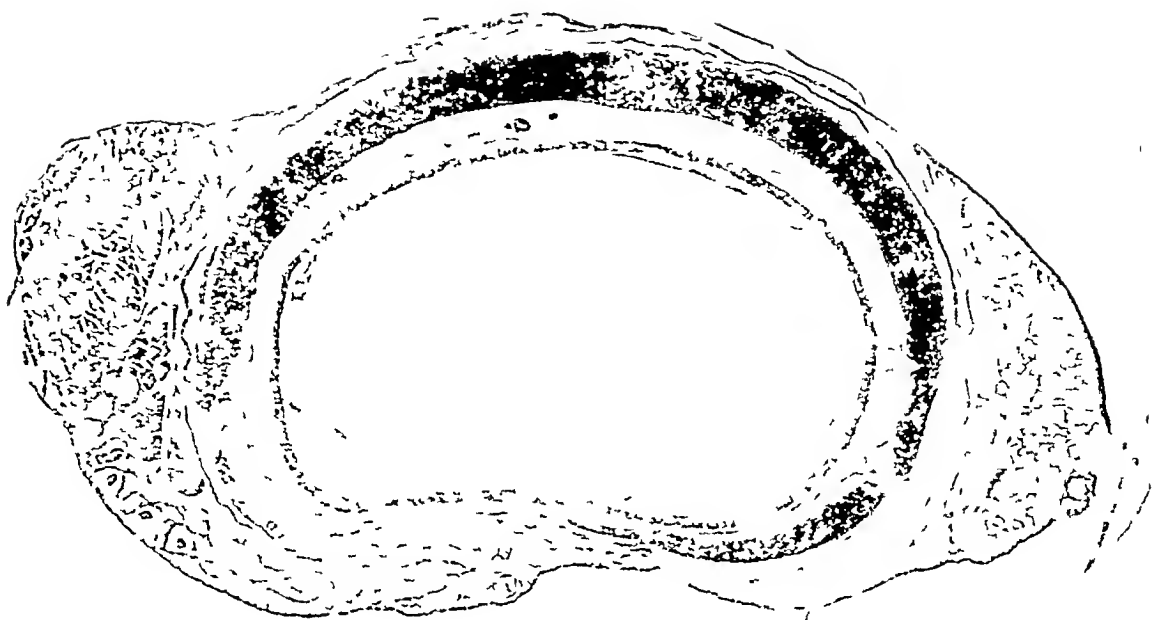


Fig 1



Fig 2

### EXPLANATION OF PLATE LII

- Fig 3 Section of trachea showing small area of round-celled infiltration forming node projecting slightly into the tracheal lumen. Note loss of columnar epithelium over node and normal appearance of ciliated, columnar epithelium in other parts of the trachea. Evidence of inflammatory reaction wanting. Magnification as in Figs 4 to 12 and 14 to 17.
- „ 4 Section showing tracheal mucous membrane. Small node composed of mononuclear cells in upper part of section, epithelium thrown into small villiform projections in lower part, engorgement of vessels and evidence of low grade inflammatory process.
- „ 5 Mucous membrane of trachea showing moderate degree of thickening, mononuclear-celled infiltration, moderate engorgement of vessels, complete absence of ciliated columnar epithelium, evidence in places of its conversion into keratinized epithelium, low grade chronic inflammatory process.
- „ 6 Mucous membrane of trachea. Chronic inflammatory and proliferative changes as in Fig 5, but causing greater thickening of mucosa. Remnants of keratinized epithelium are to be seen covering the thickened mucosa in places.

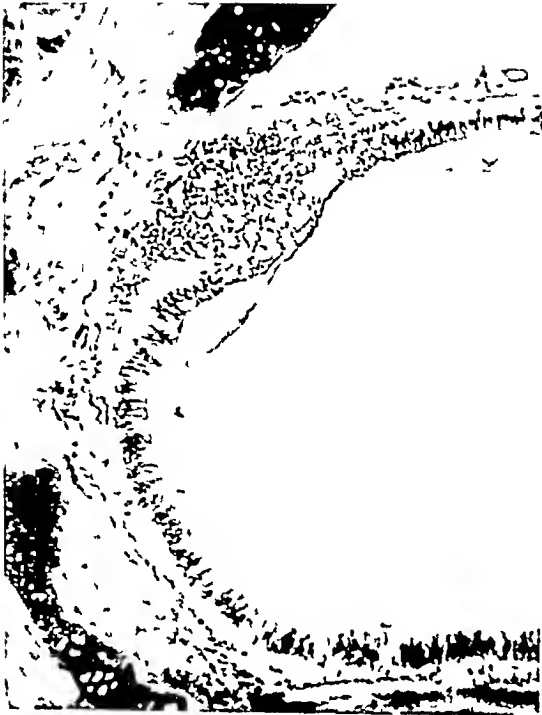


Fig 3



Fig 4



Fig 5



Fig 6

#### EXPLANATION OF PLATE LIII

- Figs 7, 8 and 9 Mucous membrane of trachea from three different cases showing increasing grades of intensity of the proliferative changes described in text. Note polypoid projections into tracheal lumen in Figs 8 and 9. In these cases evidence of inflammatory reaction was scanty.
- Fig 10 Mucous membrane of trachea showing villiform projection into the tracheal lumen, an appearance seen in only 2 of the 15 cases presenting these proliferative changes. The remaining 13 cases presented the appearances seen in Figs 3 to 9.



Fig 7



Fig 8



Fig 9



Fig 10

#### EXPLANATION OF PLATE LIV

- Fig 11 Section through the normal trachea at level of the thyroid isthmus  
Note normal thickness of the tracheal mucosa, ciliated columnar epithelium covering it and normal distribution of lymphoid cells in the sub-epithelial tissues of the mucosa, note also normal appearance of the thyroid follicles for comparison with succeeding figures
- „ 12 Section through trachea at level of the thyroid isthmus showing chronic inflammatory and proliferative changes in the mucous membrane, loss of ciliated columnar epithelium Note also the dilated follicles of the thyroid isthmus
- „ 13 Low power view of thyroid and trachea of rat showing trachea enveloped by backward and inward growth of lateral lobes of the thyroid gland In this case the tracheal mucous membrane was not markedly diseased





Fig 11



Fig 12

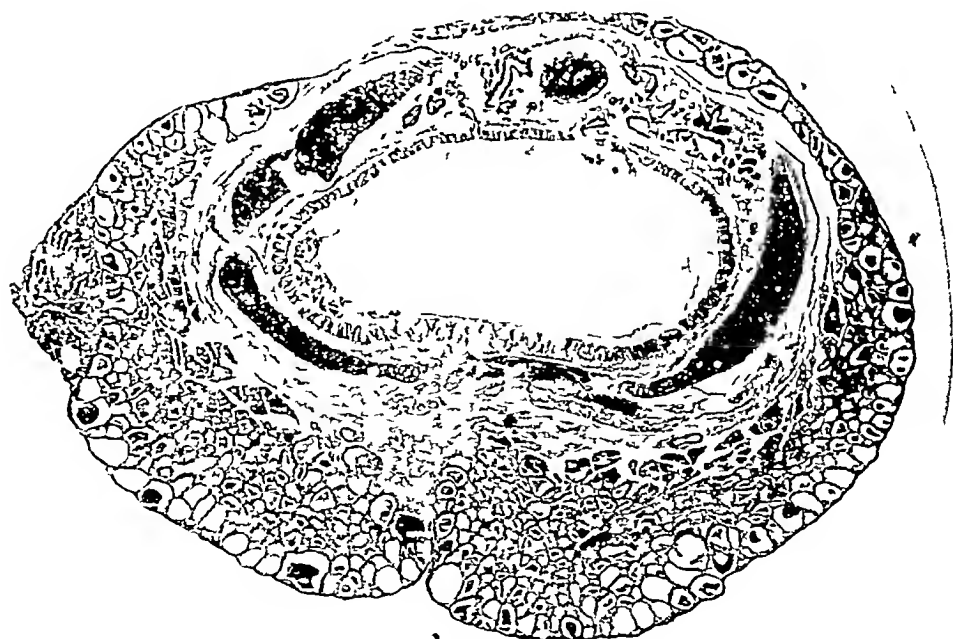


Fig 13



# OBSERVATIONS ON RAT-FLEAS AND THE TRANSMISSION OF PLAGUE

## Part I.

BY

MAJOR W J WEBSTER, M C, I M S,

AND

G D CHITRE, L M & S, B M S

(From the Haffkine Institute)

[Received for publication, June 21, 1929]

THE Plague Expert Committee of the Health Organization of the League of Nations, which met at Calcutta in December 1927, gave as one of their proposals for future study, 'Investigation of the comparative epidemiological rôle of the various species of fleas in plague transmission in selected areas of India' Experimental work in this direction has therefore been carried out in Bombay during 1928-29, with the non-pectinate rat-fleas found locally, viz, *Xenopsylla cheopis*, *X. astia* and *X. brasiliensis*

The results reported are by no means conclusive, but in view of the fact that enquiries of this nature are necessarily prolonged and liable to interruption, it has seemed desirable to issue a progress report Details of the methods adopted may be of interest to other workers on the subject and criticism will be welcomed

The analysis of the existing knowledge of the parasitology of plague by Hirst of Colombo(1) has been found an invaluable guide, and it is not proposed to give detailed references to the literature

## FLEA BREEDING

To avoid the necessity of prolonged search of rats in order to obtain a sufficient number of fleas when required for transmission experiments, it was decided to make use of laboratory-bred fleas The use of clean fleas also eliminates the risk of introducing to the experiments fleas which are already plague-infected The following method of breeding fleas has given satisfactory results, and many families of the three species have now been raised

The single transmission cage of the type used by the Indian Plague Commission is sterilized, and a layer of sterilized godown sweepings, consisting chiefly



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The single transmission cage of the type used by the Indian Plague Commission is sterilized, and a layer of sterilized godown sweepings, consisting chiefly

of rat-dung and vegetable refuse, is spread on the inner tray. Two white mice kept in the inner cage are fed daily on sterilized food. A number of fleas of the required species is added to the cage, say twenty males and twenty females. The fleas feed on the mice and lay their eggs chiefly in the litter. After ten days the litter is collected and stored in a cylindrical glass media jar (9 inches by 6 inches) with an aluminium cover. Young fleas may be found in the jar after about two weeks, and they continue to emerge from the litter for a further two or three weeks. When required for experimental purposes the young fleas are easily captured singly in test tubes, and can be sorted out into males and females with the aid of a hand lens. As neither the spermatheca of the female nor the curved rods of the sexual apparatus of the male are fully chitinized in the new-born flea, it has been found easier to distinguish the sexes by the presence of the dorsal groove on the head of the male, and its absence in the female. By preparing a family of each species once a fortnight, a constant supply of the required fleas, in moderate numbers, has been available.

#### THE MIXED FLEA EXPERIMENT

The experiments of Taylor and Chitre, carried out in this laboratory some years ago, have been criticized on the grounds that *cheopis* was apparently a more efficient transmitter between guinea-pigs than between rats, whereas transmissions with *astia* were more frequent between rats than between guinea-pigs, a discrepancy for which there is no apparent reason. Hirst suggests that the variable factor, not taken into account, was the degree of septicæmia in the animals used, and, he advises, that to compare the efficiency of different fleas as transmitters, they should be infected on the same septicæmic animal. It is desirable to ascertain the extent to which the blocking phenomenon occurs in different fleas, and the only proof of blockage is found in the behaviour of the flea when applied to feed under observation. The following method of experiment was adopted —

The single transmission cage is sterilized, and a Madras rat, inoculated cutaneously with the spleen of a known plague rat is introduced to the inner cage. Thirty-six fleas, six female and six male, *cheopis*, *astia* and *brasiliensis* are added to the cage. When the rat is found dead, the cage is emptied into a large glass bowl, and the surviving fleas are collected. The rat is examined, and if it shows well-marked signs of plague, the fleas are identified by Taylor's pipette method, and are kept individually in labelled tubes.

The technique of feeding the fleas is much the same as that described by Hirst. A rat is immobilized with the closely clipped belly uppermost; it is covered with a piece of copper gauze, lined externally with surgical lint, leaving a circular aperture about 2 inches in diameter over the clipped part of the rat. The copper gauze is secured to the platform by means of drawing pins, in such a way that the edges of the aperture fit snugly to the skin of the rat. White surgical lint, woolly side uppermost, surrounds the field of observation. This has proved less troublesome than a cotton-wool sheet, and it is an easy matter to capture escaping fleas. The feeding area is illuminated by an electric microscope

lamp with a focusing attachment, and a flask of water is interposed to intercept the heat rays. An entomological microscope on an extending arm can be swung over the rat when required. One rat is used for each group of fleas, i.e., six rats in all. Each flea in turn is applied by inverting the test tube over the exposed part of the rat. If the flea has not attempted to feed, it is removed at the end of two minutes. Occasionally the flea can be induced to bite by shading the field. Fleas which apply their biting parts are observed by swinging the microscope across. If the process of feeding cannot be readily seen, a different aspect may be viewed by rotating the platform on which the rat is fixed. The behaviour of each flea is noted. The surviving fleas are generally applied on three successive days, each group to the same rat as before.

The rats are subsequently kept in cages until they die or until the expiry of three weeks when they are killed. In either case the rats are carefully examined for signs of plague.

Fourteen of these experiments have been completed; several were abandoned because the inoculated rat did not show good signs of acute plague. A total of 529 fleas was employed, of which 438 were recovered. These were offered a total of 1,206 feeds, of which 767 were accepted. In two cases the rats died of acute plague, and in both of these the fleas fed on the rats had been male *brasiliensis*. One rat, which had been used for female *cheopis* fleas, survived for three weeks, but showed well-marked signs of resolved plague when examined post-mortem. Susceptible Madras rats, free of ectoparasites, have been used throughout.

Details of an experiment are given in Table I; it happens to be the only one in which the fleas were fed for four successive days; the consolidated figures are shown in Table II.

TABLE I  
*Details of a mixed flea experiment*

Number of experiment	Date of infection and death 'A' rat	Original number	FLEAS				TOTAL FEEDS		Death of 'B' rat	'B' rat killed	Signs of plague 'B' rat
			Sex	Species	Number recovered	Number of days fed	Offered	Accepted			
6	5-1-29 7-1-29	6	Female	<i>X cheopis</i>	6	4	24	13		28-1-29	
		6	Male	<i>X cheopis</i>	6	4	24	19			
		6	Female	<i>X astia</i>	4	4	15	8			
		6	Male	<i>X astia</i>	6	4	24	23			
		6	Female	<i>X brasiliensis</i>	6	4	23	10	14-1-29		
		6	Male	<i>X brasiliensis</i>	5	4	20	13			

TABLE II  
Total flea figures for fourteen experiments

Species of flea	Sex	Original total	Total fed	Total number feeds offered	Total number feeds accepted	Percentage of feeds accepted	Successful transmissions
<i>X cheopis</i>	Female	88	75	201	102	51	1
<i>X cheopis</i>	Male	89	80	224	151	67	
<i>X astia</i>	Female	87	70	188	120	64	
<i>X astia</i>	Male	88	60	172	145	84	
<i>X brasiliensis</i>	Female	89	78	214	100	47	2
<i>X brasiliensis</i>	Male	88	75	207	149	72	

This method of investigation is laborious in the extreme and it is obvious that no conclusion can be drawn for the results so far available. As a result of approximately 250 feeds by each species, there resulted two transmissions with *brasiliensis*, one with *cheopis* and none with *astia*. The successful transmissions with *brasiliensis* are of interest, and this species deserves more attention than it has, so far, received, considering that it forms a considerable proportion of the rat-flea population in Southern India, e.g., Cragg's figure for *brasiliensis* in Belgaum was 55 per cent.

In the case of the three successful transmissions, none of the fleas employed had been recognized as blocked and all appeared to draw blood. In some cases there was a delay before blood entered the stomach. None of these fleas defæcated in the course of the meal. In several of the negative experiments, defæcation was noted during the feed. Temporary blocking was also diagnosed on several occasions in the course of the negative experiments, i.e., the flea was seen to suck vigorously for some time before the dark proventriculus cleared suddenly, and filled with red blood.

In connection with these mixed flea experiments, some observations regarding the extent to which the various fleas can harbour the plague bacillus were carried out. At the end of each of the first six experiments the fleas were dissected and the stomach contents smeared out, stained and examined, and plague-like bacilli were seen in the majority, including representatives of all groups of fleas. In Experiment 7, smears showed fifteen out of twenty-four to contain plague-like bacilli. Samples for culture were taken from the same twenty-four and plague bacilli were grown from every one of them.

This finding has been repeated several times, a large proportion of fleas of the three species leaving a septicæmic animal and feeding in the interval still harbour plague bacilli after several days. Examination of smears of the stomach contents will demonstrate only a certain number of the infected fleas. Thus, in



Experiment 15, out of twenty-two fleas examined, four showed plague-like bacilli in smears, whereas nine of the cultures were positive, in Experiment 16, out of twenty-four fleas examined, fourteen were plague infected according to the smears, but twenty-one on culture

It would appear that the sex of the flea is a factor in plague transmission. Hirst's transmitting fleas were all females. In a series of experiments reported by Goble,(2) the transmissions were brought about by males. During the present studies, transmissions have been noted under different conditions with *cheopis* both males and females, *astia* females, and *brasiliensis* males. It may be that a larger series of experiments under any one set of conditions would abolish the apparent difference in the value of males and females as transmitters.

As laboratory-bred fleas have been used in these experiments, the question has arisen whether the 'wildness' of the flea could have any effect on the transmitting power. Strickland and Swellengrebel(3) found that the transmission of *Trypanosoma lewisi* was not so successful with laboratory-bred fleas, and they quote another example in the transmission of *T. gambiense* and the 'wildness' of tsetse flies. Laboratory-bred fleas can convey plague, but there is not sufficient evidence as to whether the power to transmit is greater or lesser than in the case of wild fleas.

#### THE PIT EXPERIMENT

This method of investigation was designed to demonstrate to what extent *X. cheopis* and *X. astia*, respectively, can carry on a plague epizootic, among highly susceptible rats under optimum conditions in Bombay. In view of the impossibility of maintaining a pure flea population in godowns, it was decided to make use of flea-proof pits to represent godowns. A rat-proof pukka-built room with a flea-proof floor of glazed tiles was made use of for the purpose. As it was necessary to know the height to which a rat can jump, and as the information was not available, a preliminary experiment was carried out. Rats were kept in the room, and the only food provided was on a ledge, the height of which could be varied. After a period of some weeks, it was decided that wild Bombay *Rattus rattus* can jump to a ledge 25 inches high but not to 26 inches. Two pits were then constructed in the room. In each case a brick wall three feet high and one foot thick enclosed a circular pit six feet across. The inner surface and the top of the wall were finished with smooth cement.

The pits were prepared for occupation by flaming the walls and floor with a painter's blow-lamp. The top of the wall was smeared with a resinous compound such as that used to coat fly papers, and thus effectually prevented the entry or exit of fleas. To prevent the resin from trickling down the inner surface, a rim of plasticine on the inner edge was found effective. Sterilized godown sweepings and straw were scattered over the floor of the pits. Cover for the rats consisted of a lidless box with the ends knocked out, and inverted to form a tunnel six inches high, in the centre of the pit.

It was arranged that the grain given as food to the rats should be sterilized before use and that the ration of green vegetable should be put in a cubical wire cage, measuring six inches which was to be dipped in boiling water for half a minute to kill any unwanted parasites or eggs. All rats added to the pits were freed of ectoparasites by the petrol method, and were subsequently kept in suspended cages for ten days so that only survivors from the petrol treatment were used. Owing to an unexpectedly large number of casualties, a shorter period than ten days was sometimes allowed. Later on, hand-picking of fleas, aided by the use of spirits of camphor, which has been found the most useful of repellants, was resorted to. The fleas added were all laboratory bred, and rats imported from Madras were used throughout. The early history of the pits is explained in Table III.

TABLE III

Date	<i>X cheopis</i> Pit	<i>X astia</i> Pit
3-1-29	12 healthy Madras rats introduced	12 healthy Madras rats introduced
17-1-29	40 <i>X cheopis</i> added, 20 males, 20 females	40 <i>X astia</i> added, 20 males, 20 females
28-1-29	Do	Do
30-1-29	20 <i>X cheopis</i> added, 10 males, 10 females	20 <i>X astia</i> added, 10 males, 10 females.
26-2-29	One guinea-pig introduced	One guinea-pig introduced
27-2-29	Guinea-pig removed and searched for fleas, 5 males, 5 females, all <i>X cheopis</i> found	Guinea-pig removed and searched for fleas, 40 males, 21 females, all <i>X astia</i> found
3-3-29	Inoculated rat introduced ('A' rat)	Inoculated rat introduced ('A' rat)
5-3-29	'A' rat dead of plague, smears showed scanty plague bacilli	.
6-3-29		'A' rat dead of plague, numerous bacilli in smears
10-3-29		First uninoculated rat dead of plague
17-3-29	Inoculated rat introduced (2nd 'A' rat)	...
18-3-29	2nd 'A' rat dead of plague, very scanty bacilli in smears and none in heart-blood	
20-3-29	Inoculated rat introduced (3rd 'A' rat)	
21-3-29	3rd 'A' rat dead of plague, scanty bacilli in heart-blood	
25-3-29	Four uninoculated rats dead of plague	.

Note.—The 'A' rats were introduced on the day following their inoculation.

Casualties in the pits were removed once a day and replaced by healthy rats. The dead rats were carefully examined for pathological signs of plague, and smears of heart-blood and spleen were examined in all cases. The results are shown in Table IV. Each rat added was given a reference number and marked for identification by a series of linear clips in the hair on either side of the

TABLE IV  
Weekly record of casualties in experimental plague pits in Bombay

Week ending	X CHLOPIS PIT				X ASFLA PIT				Temperature and Humidity		
	Total deaths	Deaths 1 to 3	DAYS IN PIT PRIOR TO DEATH FROM PLAGUE			Total deaths	Deaths 1 to 3	DAYS IN PIT PRIOR TO DEATH FROM PLAGUE			
			Max	Min	Av			Max		Min	Av
16-3-29						8	6	9	4	7.0	
23-3-29						20	17	13	3	6.6	
30-3-29	16	13	10	4	6.4	31	21	18	2	4.4	
6-4-29	28	20	6	3	3.4	36	15	6	3	3.9	
13-4-29	27	13	4	3	3.3	46	3	5	4	4.3	Av T Max 85°F Min 81°F
20-4-29	27	10	4	3	3.2	34	8	5	3	3.7	Av T Max 85°F Min 82°F
27-4-29	23	21	7	3	4.5	15	15	10	3	5.7	Av T 9 am 85°F
4-5-29	22	20	6	3	3.7	26	21	5	3	3.7	Av T 9 am 86°F
11-5-29	22	21	5	2	3.8	24	20	5	3	3.8	Av T Max 91°F S D Max 149 Min 05.4
18-5-29	21	19	5	3	4.0	15	12	6	4	4.5	Av T Max 91°F S D Max 149 Min 061
25-5-29	20	14	6	3	4.1	25	21	6	3	4.0	Av T Max 92°F S D Max 150 Min 061
1-6-29	24	15	6	2	4.3	18	15	6	3	4.1	Av T Max 92°F S D Max 163 Min 08.2

The very large mortality from causes other than plague is of interest it began on 4th April in the *astia* pit, and about a week later in the *cheopis* pit, and was associated in both cases with an enormous increase in the flea population, the rats apparently being worried to death before signs of plague had time to appear. At that period many rats survived only one day, and the majority died within two days. From 15th April an oily pulicide was used, and by judicious spraying of both pits on several occasions, a large reduction in the number of fleas was effected. The result was that the rats survived for another day or two and showed good signs of plague. The difference between the figures for the weeks ending 20th April and 27th April corresponds to the reduction in the flea population. The number of fleas did not again become excessive, but remained much higher than would ever occur in nature.

The flea population has remained remarkably pure between 3rd and 10th April, out of 844 fleas examined from the *astia* pit, there were found six *X cheopis*. At all other times there has been no contamination detected and many hundreds of fleas from each pit have been examined.

Hirst has pointed out that it is advisable to test the power of continuous transmission of the different fleas, but notes that in the tropics such a method is attended by 'very grave difficulties' owing to the danger of infestation with *Tyroglyphidae* and other unwanted parasites. The pit experiment appears to get over these difficulties, and to have proved that, as Hirst suggests, given highly susceptible animals, a virulent strain of plague and many fleas, both *astia* and *cheopis* will be found to be successful transmitters of plague. The results, however, were scarcely anticipated under the conditions indicated by the temperature and humidity figures.

To arrive at the relative efficiency of the two is a much more difficult problem. An attempt was made to get an idea of the numerical value as transmitters of the pit fleas, by removing samples of fleas, discarding those which had not had a meal, and adding batches of each sex to Madras rats in transmission cages. The results of these experiments are shown in Table V.

TABLE V  
*Transmission experiments with fleas from epizootic pits*

Date	Number of experiment	NUMBER OF FLEAS USED				Number of days before death of rat from plague
		<i>X cheopis</i> female	<i>X cheopis</i> male	<i>X astia</i> female	<i>X astia</i> male	
31-3-29	6			20		
"	7				20	
"	8	20				9
"	9		20			12

TABLE V—*concl'd*

Date	Number of experiment	NUMBER OF FLEAS USED				Number of days before death of rat from plague
		<i>X. cheopis</i> female	<i>X. cheopis</i> male	<i>X. astia</i> female	<i>X. astia</i> male	
4-4-29	10			50		
"	11			30		
7-4-29	12			72		
"	13				26	
8-4-29	14			52		
"	15				50	
25-4-29	103			50		13
"	104				50	
"	105	50				
"	106		50			
26-4-29	107			100		5
"	108				100	
"	109	100				4
"	110		100			
7-5-29	111			50		
"	112				50	
"	113	50				6
"	114		50			
9-5-29	115			50		7
"	116				50	
"	117	50				7
"	118		50			

The figures appear to indicate that the females were more capable transmitters than the males, but they do not quite justify comparison between the two species

The pits have latterly been used for the supply of plague-infected fleas. Samples of these are examined individually in the capillary tube, and those which show any evidence of plague growth in the proventriculus and oesophagus are selected for further study. So far, the results are disappointing. No completely

Blocked flea has been seen. Several moribund fleas had a dense clot in the oesophagus, but as they were not able to feed they could not transmit. Smears of these fleas showed very large numbers of plague bacilli. Many fleas showed some degree of obstruction, but this cleared with vigorous sucking. These observations are being continued.

#### RAT-FLEAS FED ON HUMAN BLOOD

Adult fleas of the three species can feed on human blood and can be kept alive for many days on this diet only. It has been stated(4) that *brasiliensis* does not readily feed on human blood, but this requires qualification. Laboratory-bred fleas have been fed daily on the fore-arm of a European (W J W) and some remarkable instances of longevity have resulted. Some of the results are shown in Table VI.

TABLE VI  
*Rat-fleas fed from both on human blood only*

Number of experiment	Date of birth of flea	Species	Sex	Length of life in days	Feeds offered	Feeds accepted
B22	4-9-28	<i>X cheopis</i>	Female	162	136	42
B23	5-9-28	<i>X cheopis</i>	Male	63	53	28
B34	18-10-28	<i>X astia</i>	Female	53	47	13
B42	5-11-28	<i>X brasiliensis</i>	Female	127	57	31
B33	18-10-28	<i>X brasiliensis</i>	Male	68	60	29

*Note*—The figures referring to the feeding of No. B42 apply to the last 60 days only, as the flea was not isolated from other females until then.

No male *astia* has been kept alive on human blood for more than a few days, although many fed readily.

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#### SUMMARY

(1) A simple method of raising families of laboratory-bred rat-fleas is described.

(2) Details are given of two improved experiments to allow of comparison of the efficiency as plague transmitters of different rat-fleas. One method deals with the artificial epizootic, the other with individual infected fleas.

- (3) Both *chicopsis* and *astia* have been found capable of keeping up epizootic plague under very favourable experimental conditions in the Bombay hot weather
- (4) Successful transmission by *X. brasiliensis* is recorded
- (5) A note on the longevity of fleas fed entirely on human blood is appended

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# DARJEELING TEAS

BY

NISHI KANTA RAY, B A,  
*Chemist, Darjeeling Municipality*

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## PRELIMINARIES

THE tea leaves (Chinese, *cha*) employed as a beverage consist of the prepared leaves or leaf buds of various species of *Thea*, such as *Thea sinensis*, *Thea bohea*, *Thea viridis*, *Thea assamica* and allied species belonging to the genus *Camellia*. Usually a piece consisting of the bud and two or three leaves below it is nipped off from the top of each shoot, the quality of the leaf drops off rapidly as we get further away from the bud. Tea is manufactured by subjecting the leaves successively to the processes of withering, rolling, fermenting and drying or firing. The mixture of leaves is next sorted into various grades. The mixture is first put through a sieve of 13 or 14-mesh and the tea which falls goes by the name of 'Broken Orange Pekoe' and is composed of fine broken leaf and 'tip', the spill goes through a second sieve of 11 or 12-mesh and the leaf which falls is composed largely of the first leaf of the shoot and called 'Orange Pekoe', the spill is next passed through a breaking machine and again sifted through a mechanical sorter, and the coarse grade resulting from the second leaf is known as 'Pekoe', 'Pekoe Souchong' is obtained from a mixture of the second and third leaves. There are also other grades such as 'Flowery Orange Pekoe' (formed by the unopened tip or bud), 'Fannings' (composed of the very small and light fragments of tea) and 'Dust' (i.e., extremely fine portions got out by sieving through a fine mesh sieve). Afterwards each grade is 'gaped' to the requisite moisture-content (about 6 per cent) so as to allow the leaf to mellow and, at the same time, to prevent the tea from becoming mouldy.

Ultimately, it is packed in air-tight cases (i.e., in lead-lined close-fitting wooden boxes) in order to retain the flavour and aroma, as tea is susceptible to moisture. Tea, as sold, is almost universally blended by mixing several definite kinds of tea with the object of securing a standard quality and flavour.

Next to water, tea is the beverage most widely in use throughout the world. The quality of the infusion from a certain variety of tea depends mainly upon four factors —

- (1) Quantity—The general rule is ‘one teaspoonful for each person and one for the pot,’ but the popular taste is for a stronger beverage
- (2) Soft water, i.e., water containing very little calcium and magnesium salts in solution extracts more from the tea than hard water
- (3) In order to get a brisk cup of tea the water should just be brought to the boil; water which has been boiling for a long time tastes flat owing to the escape of dissolved gases
- (4) The longer tea is infused, the higher is the proportion of tannic acid dissolved out; consequently, the water should not be allowed to infuse for more than five minutes

So far as the constituents of a cup of tea are concerned, the total amount of solid matter extracted in a 5-minute infusion is about 20 per cent (i.e., practically half of the total amount of solid matter which can be extracted) of the original weight of tea taken. In other words, when two spoonfuls (roughly 5 grammes) of tea are used to get one cupful of the liquor (averaging 8 ozs) and 5 minutes allowed for extraction, the solid matter amounts to about 15 grains and consists of flavour-producing substances (essential oil, etc.), caffeine, tannin, fats and protein matters, gummy substances (pectin, pectoses, etc.), certain parts of the mineral matter (the leading constituents of which are potash and phosphoric acid), etc. The colour of the infusion is due to substances grouped under the names of ‘tannin red’ and ‘tannin brown’.

The method of using tea differs much in different countries. The usual method is to take tea with milk and sugar to taste, the addition of milk is to be commended on hygienic grounds, as the albuminous matter of milk throws down some of the tannic acid of tea in an insoluble form, sugar (however small) adds to its nutritive value. In Russia, tea is drunk ‘with a liberal addition of sugar and flavouring of lemon’. In Morocco and generally throughout North America, the liquor is ‘almost saturated with sugar and strongly flavoured with mint’. ‘Some of the peoples of Eastern Europe take their tea with an admixture of rum’. The Tibetan drinks tea ‘which is really a soup or broth made by boiling tea leaves with rancid butter and balls of dough and adding a little salt and straining’. ‘In China and Japan tea is generally drunk without any other qualifying or flavouring addition’.

In tea infusion the most important ingredients upon which depend both the physiological action and the commercial value of tea are essential oil, caffeine and tannin.

- (1) Essential oil is one of the chief factors in determining the flavour, but its quantity is remarkably small (about 0.5 per cent) even in the most flavoury teas. 'It has been shown to contain acetone, methyl alcohol, and methyl salicylate which are factors in producing the aroma, but the substance responsible for the characteristic odour of tea has not been discovered'. The volatile oil 'appears to act as a cerebral and cardiac stimulant', the unpleasant effects (headache, giddiness, etc.) afflicting those who indulge in large quantities of tea are attributed to it.
- (2) Caffeine is the principal stimulating material from which tea obtains its most valued properties. This alkaloid satisfies some craving of the human system. It is a stimulant to the nervous system and acts on the heart also in small doses as a tonic, in excessive or too frequent doses it makes its action irregular and weak and tends to nervous depression.
- (3) Tannin causes pungency, and when changed by fermentation, gives colour to the infusion of tea. But tannin in the infusion is the undesirable part of tea owing to its astringent properties, especially in large doses it impairs digestion and impedes the action of the bowels.

'The effect of the use of tea upon health has been much discussed. The product, if carefully converted into a beverage and used in moderation, should be harmless to all normal human beings' and 'offers a nervine stimulant at a time when the brain is jaded with work'. Even in health the evil effects (e.g., insomnia, giddiness, heart trouble, nervousness, indigestion, etc.) are evident when tea is taken in excess or when it is improperly prepared. The irritating action on the stomach is least 'when the stomach is neither quite empty nor too full, but contains a moderate amount of easily digested food—a state of things which is pretty well true at "afternoon tea".'

#### SPECIALITY OF DARJEELING TEAS

The quality of tea from any area is dependent upon the combined effect of several factors such as climatic conditions, special character of soil and manuring, type of bush grown, kind of pruning, method of plucking and system of manufacture. Even when other things are equal, climate and soil vary widely from district to district and conditions under which tea is manufactured are different in every factory. As a result, the quality varies at different places, and at one and the same place at different times of the year. Teas produced at the end of September are the finest and well known for the Autumn flavour.

The Darjeeling tea gardens are situated within the Terai (a low-lying strip stretching along the base of the hills), on the lower hill slopes and higher hills rising to 7,000 feet or over in elevation. The interior of the district is 'a confused labyrinth of ridges and valleys,' varying in elevation and aspect. In

connection with the climate of the hills it should be noted that the higher we go the colder and rarer the air becomes, and its absolute humidity decreases. Owing to the physical configuration, the rainfall varies greatly in different parts. The aspect influences the subsidiary factors such as sunshine, etc. At Darjeeling, however, the sky is more or less clouded during the greater part of the year. The soil in the Terai 'is composed of alluvium, a light sandy loam being most common'. 'In the hills the greater portion of the underlying rock consists of what is known as Sikkim gneiss. The constituents of the gneiss occur in varying proportions, and the soil varies in the same relation'. The medium and 'high-grown' teas, particularly the latter, are distinctly better than those produced at lower elevations and famous for the peculiarly fine, much-sought-after flavour which is attributed mostly to particular climatic conditions, and partly to the peculiar character of the particular soils as well. Elevation has a great deal to do with the production of flavour. 'The finest teas are produced at high elevations in Darjeeling and Ceylon'. Increase in flavour is due to formation in greater quantity of the substances yielding aroma and very possibly related to lowering of the temperature during growth. Dr Harold H. Mann, D.Sc., late of the Indian Tea Association, put forward the theory—'Let there be any slightly unfavourable conditions, such as the attack of the green fly, cold weather insufficient to stop growth altogether, or the like, and the reserves, if I may so speak of the plant, are called up in defence. These reserves, in part, consist in a larger secretion of essential oil, and hence a flavoury tea is produced'. Flavour depends not only on the leaf (which varies in composition as they are plucked from the bushes at different elevations and in different parts of the district) but also on chemical changes (influenced by atmospheric conditions, notably temperature and humidity) taking place during the process of fermentation. Flavour-producing substances are however so very minute that no analytical work has so far been carried out.

Besides the question of flavour, the chief ingredients of tea are caffeine and tannin, and the main object in undertaking the work on 'Darjeeling teas' has been to determine the actual amounts of these two substances (as obtained under tea-drinking conditions) in the 'medium grown' and 'high grown' teas of this area. All available grades of tea from ten gardens in different parts of the district and at different heights were examined. As the strength of the liquor varies with individual taste, the amounts of caffeine and tannin extracted in tea-taster's infusion have been determined. Taster's infusion is made in the following manner—Four lots each of three grammes of tea are separately extracted for 5 minutes with 120 c.c. of water which has just boiled, the liquor is drained off through a funnel containing a little glass wool and the combined liquor from the four lots is diluted up to 500 c.c. This 500 c.c. of the liquor contains, therefore, the caffeine, tannin, and other soluble matter extracted from 12 grammes of tea. Taster's infusion has been made with dried tea (i.e., tea dried to a constant weight at 100°C) using Darjeeling water which is peculiarly soft, in fact almost like distilled water (total hardness varying from 0.5—1.0 parts per 100,000) and

the percentages of caffeine and tannin extracted are shown in the following Table —

TABLE I  
(The results are expressed as percentages of dry tea)

* Date of examination	Name of Garden	Grade	Per cent Caffeine (N × 3.464) latlock & Thom- son's Process		Per cent Tannin (Gallotannic acid) Lowenthal-Proctor Method	
January 1927	Happy Valley Tea Estate	Flowery Orange Pekoe (F O P)	170		3.30	
		Orange Pekoe (O P)	164		3.12	
		Broken Orange Pekoe (B O P)			3.30	
		Broken Pekoe (B P)	168		2.60	
		Orange Pekoe Dust (O P Dust)	206		4.30	
		Average		18		3.5
		Average excluding dust		167		3.1
Do	Ringneet Tea Estate	O P	139		3.20	
		Pekoe	158		3.12	
		Pekoe mixture	204		4.50	
		Ground Pekoe	166		2.94	
		Average		167		3.4
February 1927	Ging Tea Estate	B O P	152		2.77	
		O P	178		3.96	
		Pekoe	202		5.04	
		B P	20		4.85	
		Pekoe Souchong	168		4.42	
		Broken Tea	216		5.20	
		Average		19		4.4
5-6-27	Badamtam Tea Estate	F O P	190		4.33	
		O P	198		6.24	
		Pekoe	214		5.02	
		Souchong	212		5.20	
		Dust	230		5.89	
		Average		22		5.3
		Average excluding dust		20		5.2
3-7-27	Lopchu Tea Estate	F O P	188		3.81	
		O P	170		3.81	
		B O P	229		4.16	
		Pekoe	196		4.33	
		Pekoe Fanning	156		4.16	
		Pekoe Souchong	172		4.68	
		Dust	206		4.85	
		Average		19		4.3
12-7-27	Gielle	Average excluding dust		19		4.2
		F O P	14		2.3	
		O P	16		2.3	
		B O P	18		3.3	
		Average		16		2.6

\* All the samples for examination were received during January and February 1927

connection with the climate of the hills it should be noted that the higher we go the colder and rarer the air becomes, and its absolute humidity decreases. Owing to the physical configuration, the rainfall varies greatly in different parts. The aspect influences the subsidiary factors such as sunshine, etc. At Darjeeling, however, the sky is more or less clouded during the greater part of the year. The soil in the Terai 'is composed of alluvium, a light sandy loam being most common'. 'In the hills the greater portion of the underlying rock consists of what is known as Sikkim gneiss. The constituents of the gneiss occur in varying proportions, and the soil varies in the same relation'. The medium and 'high-grown' teas, particularly the latter, are distinctly better than those produced at lower elevations and famous for the peculiarly fine, much-sought-after flavour which is attributed mostly to particular climatic conditions, and partly to the peculiar character of the particular soils as well. Elevation has a great deal to do with the production of flavour. 'The finest teas are produced at high elevations in Darjeeling and Ceylon'. Increase in flavour is due to formation in greater quantity of the substances yielding aroma and very possibly related to lowering of the temperature during growth. Dr Harold H. Mann, D.Sc., late of the Indian Tea Association, put forward the theory—'Let there be any slightly unfavourable conditions, such as the attack of the green fly, cold weather insufficient to stop growth altogether, or the like, and the reserves, if I may so speak of the plant, are called up in defence. These reserves, in part, consist in a larger secretion of essential oil, and hence a flavoury tea is produced'. Flavour depends not only on the leaf (which varies in composition as they are plucked from the bushes at different elevations and in different parts of the district) but also on chemical changes (influenced by atmospheric conditions, notably temperature and humidity) taking place during the process of fermentation. Flavour-producing substances are however so very minute that no analytical work has so far been carried out.

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The following Tables show what may be expected from other teas —

\* TABLE II

(The analyses were made on the taster's infusion and the figures represent percentages of the leaf used)

	Number of analyses	Per cent, Caffeine	Per cent, Tannin
Dooars tea	2	2.69—3.01	4.67—5.57
Assam tea (B O P)	4	2.72—3.01	4.70—5.80
Assam tea	9	1.22—4.95	5.15—6.81
Assam tea	9		5.13—10.15

\* The figures were supplied by Mr C J Harrison, Chemist of the Indian Tea Association

TABLE III

	Per cent, Caffeine (total)	Per cent, Tannin (total)
(a) Chinese tea	2.15—3.50	7.30—10.90
(a) Ceylon tea	1.95—3.60	10.10—14.00
(a) Japanese tea	2.22—2.81	14.29—15.08
(a) Indian tea	1.95—3.45	13.30—15.00
(b) Indian tea	1.80—3.30	13.04—18.86
(c) Ceylon tea (6 samples)		6.12—11.71
(c) Assam tea (6 samples)		11.12—12.53
(d) Assam tea (25 samples)		6.66—15.11
Darjeeling tea (48 samples)	1.76—3.10	4.23—11.72

(a) 'The Chemical Analysis of Foods'—H E Cox

(b) 'Food Inspection and Analysis'—A E Leach

(c) 'Tea in Ceylon'—C R. Harler *Indian Tea Association Quarterly Journal*, Part IV, 1924

(d) The figures were supplied by Mr C J Harrison, Chemist of the Indian Tea Association

The important facts derived from the analyses of Darjeeling teas are the relatively low caffeine and tannin contents, particularly the latter. On referring to Table I, it will be seen that 1.39—2.3 grammes of caffeine and 2.3—6.24 grammes of tannin are extracted from 100 grammes of dried tea. Assuming the moisture-content to be 6 per cent (which is always aimed at in order that the tea may keep best), 1.3—2.2 grammes of caffeine and 2.2—5.9 grammes of

tannin are extracted from 100 grammes of undried tea, or 0.157—0.26 grammes of caffeine and 0.26—0.7 grammes of tannin are extracted from 12 grammes of tea in 500 cc, i.e., in taster's 5-minute infusion, or 0.07—0.117 gramme of caffeine and 0.117—0.315 grammes of tannin are extracted in 225 cc from 5.4 grammes of tea after infusing for 5 minutes. In other words, when tea is infused for 5 minutes with boiling water and the strength of the liquor corresponds approximately with that obtained in taster's infusion (roughly 2 spoonfuls for every 8 oz.), a cupful of tea (averaging 8 oz.) contains  $1-1\frac{3}{4}$  grains of caffeine and  $1\frac{3}{4}-5$  grains of tannin, the average amounts being  $1\frac{1}{2}$  grains of caffeine and 3 grains of tannin, as compared with approximately  $1\frac{3}{4}$  grains and 4 grains respectively obtained by using, under similar conditions, some of the best blended teas in the market, namely Lipton's and Brooke Bond tea. These (Darjeeling) teas are therefore well adapted for drinking alone.

So far as the effect of climate on the tannin-content is concerned, it may be stated that 'a combination of weather conditions, such as high humidity and hot, fairly sunny days, which induces sudden rapid growth, at the same time lowers the tannin content of the leaf and also the total soluble solid content. Cool weather, during which growth is slowed up, results in an increase in tannin content and general improvement in quality, although, if as often happens, these conditions are accompanied by cloudy skies, the effect of the shade is to lower the tannin content.' 'In dull, overcast weather teas lacking in pungency are produced'—*The Manufacture of Tea in North-East India*—P. H. Carpenter and C. J. Harrison.

### CONCLUSION

The chemistry of tea has not yet reached the art of the taster. The quality of a sample of tea and its commercial value can only with accuracy be determined by tasting by an experienced palate, i.e., by a skilled tea taster. The taster values a tea on appearance of leaf (both before and after it is infused) as well as on the liquor. Flavour, colour, pungency, briskness, strength are all desirable characteristics of the liquor. Pungency denotes the astringency due to the presence of tannin. Briskness is an impression of 'liveliness' as opposed to flatness, a rough measure of briskness is given by the total tannin. Total soluble solids including tannin give a rough measure of 'strength,' the gummy substances increasing the thickness of the liquor.

So far as tasting by the palate is concerned, the teas containing the most tannin usually have the greatest value, unless possessed of less flavour, and a tea may be highly valued in consequence of its outstanding flavour.

Generally speaking, in the language of the tea taster,

Darjeeling teas are brisk and give light liquor, they do not possess the strength of the average Dooars or Assam teas, but have characteristic flavour,

Assam teas possess pungency but little flavour,

Dooars teas—are noted for strength,



Chinese—teas lack in body and are devoid of any marked astringency, but have delicate flavour,

The high country teas at Ceylon—are noted for their flavour, although the liquors are light

In North-East India the highest prices naturally go to teas from Darjeeling the district *par excellence* where flavoury teas are turned out. Usually the characteristic flavour of Darjeeling teas constitutes their main attraction. The relatively low tannin-content is also a speciality of these teas from hygienic points of view. Darjeeling teas compare very favourably with the Chinese teas which are well known for being relatively poor with respect to tannin and are often prescribed in the case of constipated persons instead of the more astringent varieties. In fact, some gardens produce teas which are perhaps least astringent (i.e., of the lowest tannin-content), and preference should be given to these teas, if tea is taken at all, in cases where the digestion is enfeebled, and even then they should be infused for as short a time as possible, and the infusion detannated to some extent by using milk.

My thanks are due to Dr D. A. Farquharson, M.B.C.M., D.P.H., etc., Medical Officer of Health, Darjeeling Municipality, for valuable assistance in carrying on the work.

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opening the body Any blood in the heart was brushed away with damp cotton-wool and the heart quickly weighed to the nearest centigram A piece was then cut off from the ventricular muscle (avoiding the band of obvious oedema which often showed between the auricles and the ventricles) and dropped into a tared, stoppered, weighing bottle and weighed at once to the nearest milligram The time from opening the body to putting the sample of heart muscle in the weighing bottle was  $3 \pm 1$  minutes

The sample of ventricular muscle after weighing was covered with absolute alcohol, and after a short soaking cut with scissors into small pieces not more than 1 mm cube in size The scissors and forceps were washed into the weighing bottle with more absolute alcohol making about 5 c.c. in all The muscle was left in the alcohol till next morning and the alcohol then evaporated off in a water oven at about  $90^{\circ}\text{C}$  Fresh alcohol was then poured on, and 24 hours later this again evaporated The muscle was then dried during the next four days for a total of 18 hours in an air oven at  $100^{\circ}\text{C}$  to  $105^{\circ}\text{C}$  during the day, and in a vacuum desiccator over a calcium chloride during the night Weighings were made after 3 hours, 13 hours, and 18 hours drying in the oven, the weighing always being done in the mornings after a night in the vacuum desiccator After 13 hours in the air oven, the loss of weight was very small and the mean of the weighings after 13 and 18 hours was taken for the final weight

#### CONTROLS OF THE METHOD

Control analyses using normal pigeons not included in the Batches I and III gave the following results —

- (1) Duplicate analyses, using two or more slices from the same heart, agreed, always to within 2 mg. in the final weighings and generally to within 1 mg. As the pieces of heart muscle taken for analysis weighed from 1 to 2 grammes, the error in the percentage of water found was always less than 0.2 per cent in duplicate analyses
- (2) The difference in the water-content of different regions of the same ventricles is within the experimental error
- (3) The effect of variations in the time between opening the body of the pigeon and weighing the sample of ventricular muscle, up to 10 minutes, is negligible

As many of the deficiently fed pigeons died in the night and lay dead for varying and unknown periods before coming to post-mortem examination, the control pigeons also were allowed to lie for varying periods after being killed before their hearts were taken out, to see if this affected the water-content of their heart muscle In 13 birds the heart was taken out at once, i.e., within 10 minutes of death, in 12 birds half an hour was allowed before opening them, in 10 birds 4 hours, and in 13 birds 6 hours The results are shown in Table I It will be noticed that although the mean water-content of the ventricular muscle rises as the time after death increases, the differences are too small to be statistically significant

TABLE I

*Showing the effect of post-mortem changes on the water-content of the heart muscle*

	TIME FROM DEATH TO REMOVAL OF THE HEART				All controls
	10 minutes	Half an hour	4 hours	6 hours	
Number	13	12	10	13	48
Mean body-weight at death	289	301	318	290	298
Mean water content of ventricular muscle	75.98	76.11	76.13	76.45	76.16
Standard deviation	0.74	0.40	0.47	0.47	0.57
Standard error of the mean	0.21	0.12	0.15	0.13	0.08

TABLE II

*Showing the general results of the experiments*

Statistic	CONTROLS			DEFICIENTLY FED PIGEONS					Diff 8-3	St error of diff
	Batch I	Batch III	All	Batch II	Batch IV	Batch V	Batch VI	All		
	1	2	3	1	5	6	7	8		
Diet	Mixed grains			Rice plus chl		Rice plus ragi				
Numbers	24	24	48	20	24	24	24	92		
Mean length of life in days				36.0	37.1	51.7	49.8	44.0		
Standard deviation				11.4	8.3	21.5	12.9			
Mean original body-weight	291	310	301	291	319	270	286	292	-9	4.8
Standard deviation	27	22	26	27	27	12	20	29		
Mean final body-weight	289	307	298	175	172	178	170	174	-124	4.0
Standard deviation	21	22	23	20	19	24	21	21		
Mean heart-weight	3.23	3.35	3.29	2.34	2.26	2.31	2.16	2.27	-1.02	0.09
Standard deviation	0.51	0.44	0.48	0.59	0.45	0.52	0.41	0.50		
Mean ratio heart-weight/original body-weight $\times 10^3$	11.2	10.8	11.0	7.8	7.1	8.5	7.7	7.8	-3.2	0.31
Standard deviation	2.0	1.4	1.7	1.9	1.2	1.8	1.6	1.7		
Mean water content of ventricular muscle	76.2	76.2	76.2	79.0	79.3	78.7	79.4	79.1	+2.9	0.20
Standard deviation	0.7	0.5	0.6	1.9	1.8	1.8	1.4	1.8		

## RESULTS

The complete data for all the pigeons are given in the Appendix, and for convenience certain statistics have been calculated from them and are given in Table II

*Length of life*

The control pigeons (Batches I and III) all remained healthy and were killed at various periods from 17 to 53 days from the beginning of the experiment

Amongst the deficiently fed birds, four (in Batch II) refused to eat and died of pure starvation shortly after being put on the diet. These have been excluded from the calculations. The remaining birds survived for various periods up to 81 days. The average length of life in the different batches is shown in Table III

TABLE III

*Showing the average length of life in the batches of deficiently fed pigeons*

Batch	Addition to basal diet	LENGTH OF LIFE IN DAYS			Standard deviation
		Mean	Range		
			Lowest	Highest	
II	0.8 gr dal	36.0	21	60	11.4
IV		37.1	26	62	8.3
II and IV		36.6	21	62	9.7
V	2 grs ragi	51.7	19	81	21.5
VI		49.8	27	79	12.9
V and VI		50.8	19	81	17.8

*Body-weights*

The mean weights of the various batches as the experiment progressed is shown in Table IV

TABLE IV

*1 m. loss of body-weight in the deficiently fed pigeons as the experiment progressed*

1 to 2 g

less than 0.5 per

(2) The difference in the AND MEAN BODY-WEIGHT OF THE SURVIVORS

(3) Variations in the

As many  
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Batch IV	Batch V		Batch VI	
	Nos	Wt	Nos	Wt
24	24	270	24	286
24	24	276	24	272
21	21	251	24	267
22	22	228	24	250
18	18	213	23	232
16	16	218	21	222
15	15	217	17	216
4	4	207	12	205
1	1	204	8	188
		198	3	168
		195	1	155
		170	1	150

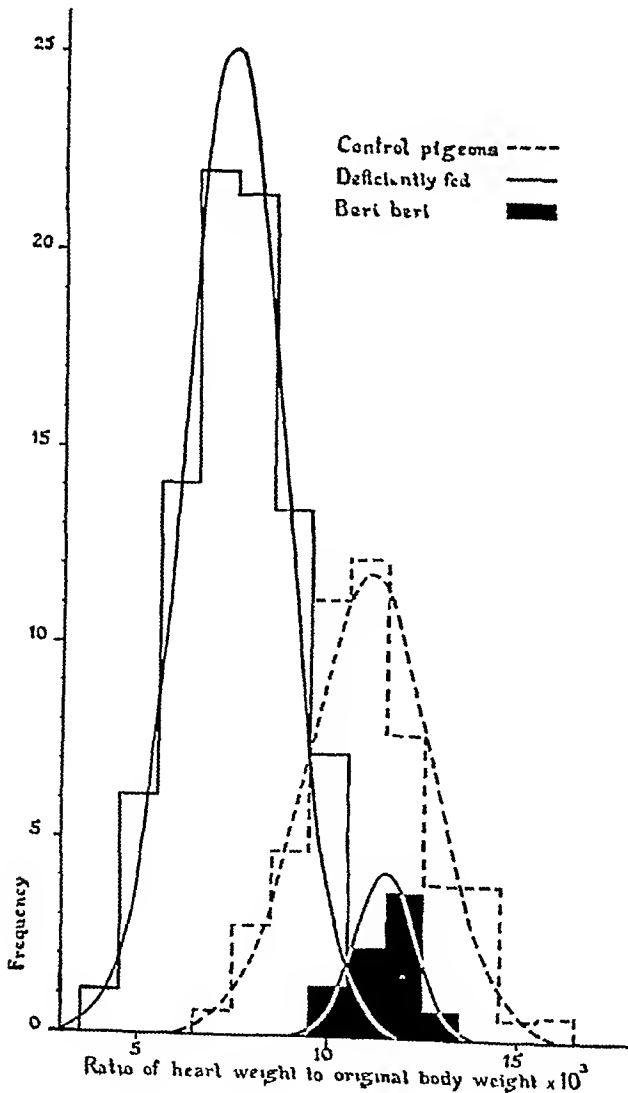
### Symptoms

Symptoms of polyneuritis were generally observed before death but not invariably, and more commonly amongst those pigeons receiving an addition of dal than amongst those receiving an addition of ragi. Most of the deficiently fed pigeons died in the night so that the symptoms of polyneuritis, if of but short duration, may have frequently escaped observation.

### Heart-weights

As the heart-weight in pigeons depends on, and is closely correlated with, the body-weight, the ratio of the heart-weight to the original body-weight, multiplied

Diagram I



A comparison between the statistics calculated for normal pigeons, pigeons suffering from polyneuritis columbarum and pigeons suffering from beri-beri columbarum is given in Table V

TABLE V

*Showing a comparison between the normal, polyneuritis and the beri-beri pigeons*

Statistic	Normal	Polyneuritis columbarum	Beri-beri columbarum	Significance
Numbers	48	84	8	
Mean length of life in days		44	43	Not significant
Mean original body-weights	301	293	275	Not significant
Mean final body-weight	298	176	198	Probably significant
Mean heart-weight	3.29	2.18	3.16	Significant
Mean ratio of heart-weight to original body-weight $\times 10^3$	11.0	7.4	11.5	Significant
Mean water-content of heart muscle per cent	76.2	79.2	77.7	Probably significant

In the last column are given the statistical significances of the differences between the figures for the polyneuritis and beri-beri pigeons

## SUMMARY

The effect of a beri-beri diet on the heart of pigeons are —

- (1) In the majority of the birds, a diminution in the size of the heart and an increase in the water-content of the heart muscle (polyneuritis columbarum and starvation)
- (2) In a minority of the birds, an increase in the size of the heart, and some increase in the water-content of the heart muscle (beri-beri columbarum). This increase is, however, certainly not greater than, and probably less than, in other pigeons, suffering from the effects of the diet
- (3) The large heart of beri-beri columbarum is not to be explained by water-retention

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## APPENDIX

In the following tables the complete data are given for all the 140 pigeons used in these experiments, with the exception of the average body-weights of the survivors. These have already been given in Table IV for the deficiently fed pigeons. The control pigeons maintained their original body-weights, within small variations.

## BATCH I

*Control pigeons on a diet of mixed grains*  
*Experiment started 25th September, 1928*

Day of the experiment.	Original body-weight	Final body-weight	Heart-weight	Water-content of heart muscle
29	300	295	4.23	76.8
29	220	270	2.97	76.9
29	320	305	2.40	76.1
29	290	285	2.85	75.6
34	320	300	2.90	75.9
34	285	275	2.95	74.9
36	260	250	2.70	74.6
36	280	270	2.56	75.4
38	265	300	2.69	75.8
38	265	295	2.81	75.3
39	315	305	2.75	76.6
39	315	305	3.37	76.7
39	325	300	3.28	76.7
42	290	310	3.20	75.4
42	315	310	3.18	76.4
43	315	275	3.69	76.9
43	300	300	3.86	76.9
50	300	275	3.71	76.6
50	300	300	2.89	75.9
52	250	250	3.90	76.0
52	280	280	3.94	76.2
52	255	250	3.49	76.6
53	325	330	4.06	77.1
53	290	300	3.27	76.4

## BATCH II

*Diet milled rice ad lib plus 0.8 gramme dal per pigeon per day*  
*Experiment started 25th September, 1928*

Day of the experiment	Original body-weight	Final body-weight	Heart-weight	Water-content of heart muscle
21	320	180	1.79	78.5
21	280	195	2.39	76.9
22	300	185	2.94	79.2
24	300	220	2.34	75.8
27	315	175	2.30	78.3
28	265	205	3.20	76.2
31	290	155	1.85	81.4
32	315	185	2.91	78.6
32	315	185	2.20	79.0
32	320	180	2.32	78.8
35	280	165	1.86	78.3
36	325	180	2.66	80.8
38	315	175	2.07	75.1
41	285	165	2.22	78.3
42	300	145	1.84	81.6
42	290	150	1.49	80.2
49	260	155	1.92	80.2
49	290	140	1.86	81.9
58	325	190	4.08	80.1
60	300	175	2.55	80.4



## BATCH III

*Control pigeons on a diet of mixed grains*  
*Experiment started 20th November, 1928*

Day of the experiment	Original body-weight	Final body-weight	Heart-weight	Water-content of heart muscle
17	300	295	3.68	77.0
17	310	290	3.60	76.5
44	335	325	3.24	76.4
44	305	320	3.55	75.8
44	280	270	2.77	76.5
44	275	320	3.15	75.4
44	285	275	3.05	76.6
44	325	295	2.56	75.6
45	290	285	2.82	75.8
45	335	310	2.92	76.3
45	320	310	3.50	75.7
45	300	310	3.58	76.3
45	305	300	3.08	76.5
45	285	290	3.02	76.4
51	315	345	3.84	76.3
51	295	270	2.38	76.1
51	300	320	4.15	75.8
51	310	290	3.38	75.7
51	330	315	3.77	77.1
51	330	325	3.65	75.9
52	305	315	3.83	76.2
52	305	325	3.50	75.6
52	375	360	3.85	75.8
52	315	315	3.48	76.8

## BATCH IV

*Diet milled rice ad lib plus 0.8 gramme dal per pigeon per day**Experiment started 30th October, 1928*

Day of the experiment	Original body-weight	Final body-weight	Heart-weight	Water-content of heart muscle
26	290	155	2.14	78.4
26	310	220	2.37	76.4
27	270	165	1.95	77.8
30	370	195	2.83	75.8
30	300	155	2.19	79.9
30	300	145	1.33	79.9
32	340	185	2.70	77.0
32	290	155	1.75	77.3
32	300	170	2.67	79.4
35	300	140	1.35	80.2
35	290	165	2.31	82.0
35	315	175	2.23	78.6
36	325	155	1.92	78.8
38	315	160	2.27	81.4
38	315	185	2.53	80.4
39	350	210	3.17	79
39	345	195	2.46	78
39	350	2	2.11	"
40	300		1.68	
41	290		2.09	
48	340		2.67	
50	340		2.1	
51	340			
62	370	1		

## BATCH V

*Diet milled rice ad lib plus 2 grammes yagi per pigeon per day**Experiment started 18th February, 1929*

Day of the experiment	Original body-weight	Final body-weight	Heart-weight	Water-content of heart muscle
19	285	225	3.31	77.0
21	280	195	2.50	80.2
21	275	195	2.45	79.4
24	270	185	2.57	78.4
26	290	185	2.89	78.4
27	260	195	2.77	76.5
31	270	165	2.06	76.3
34	265	155	1.82	78.9
40	255	155	1.95	79.7
46	250	160	1.68	75.8
51	290	150	1.62	78.7
52	260	240	3.09	75.3
55	280	170	1.98	79.6
57	270	200	2.71	79.3
62	260	160	2.48	79.5
68	285	175	2.63	81.6
68	280	180	2.11	79.6
72	275	200	3.28	79.2
74	270	180	2.43	79.5
76	250	160	1.95	77.9
77	280	165	1.63	79.7
79	255	145	1.63	82.7
81	255	145	1.80	78.4
81	270	175	1.99	77.6

## BATCH VI

*Diet milled rice ad lib plus 2 grammes ragi per pigeon per day**Experiment started 2nd March, 1929*

Day of the experiment	Original body-weight	Final body-weight	Heart-weight	Water-content of heart muscle
27	275	205	3 00	77.4
29	270	155	1 97	77.7
34	270	175	2.23	79.0
36	285	175	2.43	79.9
36	295	150	2 13	78.9
38	285	175	2.52	
40	285	190	2.26	78.1
43	290	195	1 99	78.5
45	330	195	1.99	79.1
46	260	155	2.11	79.7
47	315	200	2.48	81.5
47	280	200	2.79	79.0
54	270	160	2.34	79.2
54	305	210	2.59	79.8
54	285	160	1 53	77.8
56	295	165	1 77	78.0
57	270	155	1.40	77.7
58	270	160	1 75	79.6
59	250	125	2.55	80.2
62	305	175	1 58	82.6
63	275	150	1 79	79.2
66	320	150	2.53	81.5
66	315	160	1 69	81.0
79	260	150	2.45	81.1

# A SCHEME FOR THE ANALYSIS OF SMALL URINARY CALCULI

BY

MAJOR CLIVE NEWCOMB, D M, F I C, I M S,  
*Nutritional Research, I R F I Coonor, S India*

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IN the course of the investigations into the composition of vesical calculi in India in progress in this laboratory, the need was felt for a scheme of analysis which would be applicable to very small stones, as some of the human stones and all the stones which occurred in rats were too small for analysis by usual methods

The scheme detailed in this paper was devised and tested first against pure substances and then against small quantities of stones of which enough was available for the usual macro methods. The results on the micro scale are in general not so accurate as when enough stone is available for the usual macro methods—as is to be expected—but accurate enough for a comparison of the composition of stones from different parts of India. The results on this micro scheme of analysis are generally within 1 per cent of the results of the macro methods

## PRINCIPLE

The stone or stones are powdered and dried. Of the dry powder part is wet ashed as for a Kjeldahl determination and the ash dissolved in normal sulphuric acid. Portions of this solution are then taken for the determination of phosphates, total nitrogen, calcium and magnesium. Fresh portions of the dry powder are taken for the determinations of oxalates and carbonates (if present), and for qualitative tests for uric acid (if nitrogen is low), cysteine, creatinine, and any other substances which are suspected to be present. A complete analysis can be made with 30 milligrams of stone, but three times this amount is better if available

## DETAILS

1 *Sampling*—The whole of the stone or stones are powdered in an agate pestle and mortar

The procedure is, therefore, different according as more than a trace of phosphates are present or not

If no, or only a little, phosphate is present, 5 c c of the stone solution are measured into a 50 c c flask and roughly 30 c c of water added. The solution is then nearly neutralized by the addition of 4 c c of normal sodium hydroxide, 2 c c of Nessler's solution added and the whole made up to bulk. The colour is read against a standard ammonium sulphate solution with like quantities of reagents

The colour, if deep, is best read in a colorimeter, but, if faint, in Nessler glasses

If phosphates are present in any considerable quantity, ten c c of the stone solution are made alkaline with sodium hydroxide, the solution allowed to stand for a little while to give the precipitate of phosphates time to form and the whole then centrifuged. Of the clear supernatant liquid, 5 c c are taken and the nitrogen in this determined as before

The following controls (*vide* Table II) were made of this method of determining the total nitrogen, using stones in which the total nitrogen had also been done by an ordinary macro-Kjeldahl's method, with distillation of the ammonia into a standard acid and back titration of the acid used —

TABLE II

Stone number	PERCENTAGE OF NITROGEN FOUND		Difference
	Macro	Micro	
120	21.8	22.0	+0.2
58	30.1	30.7	+0.6
100	26.6	28.4	+1.8
103	29.4	29.0	-0.4
121	2.1	2.2	+0.1
130	0.8	Trace	
39	31.8	30.7	-1.1
82	12.3	11.3	-1.0
79	17.9	17.9	0
157	3.2	3.3	+0.1
28	32.9	31.9	+1.0
Average difference			±0.57

#### *Calcium determination*

The determination of calcium as phosphate as recommended by Briggs(1) was first tried but in our hands did not give satisfactory results. The following

method was adopted—ten cc of the stone solution are measured into a test tube, ten drops of saturated ammonium oxalate solution added, and one drop of methyl red. Strong ammonia is then added, drop by drop, till the colour changes to yellow, and then very dilute acetic acid, drop by drop, till the colour changes to brown (pH 5.3). The tube is heated in a water-bath, cooled and allowed to stand till next day, and then the precipitated calcium oxalate filtered off through a Whatmann No. 44 paper. The precipitate is washed carefully with water containing a few drops of ammonia, not more than about 10 cc of wash water being used. This is sufficient to get rid of practically all the excess of ammonium oxalate if the washing is done with care, using a dropping pipette. The filtrate and washings are preserved for the subsequent magnesium determination (*q.v.*). The calcium oxalate is then dissolved in normal sulphuric acid and the filter paper washed, again using about 10 cc. The solution is then warmed and titrated with N/100 permanganate solution using a micro burette which can be read to 1/100ths of a cc (1 cc of N/100 permanganate = 0.28 mgs of CaO). Each time a set of determinations is done, it is necessary to do a blank experiment in which normal sulphuric acid is used instead of the solution to be analysed. This is treated exactly as the others and the filtrate and washings preserved to provide a blank for the subsequent magnesium determination (*q.v.*). The amount of permanganate required for this blank is then deducted from the amounts for the solution to be analysed. For example, the following determinations (*vide* Table III) were made using a solution of calcium carbonate which contained 0.67 milligram of CaO per cc as estimated by a similar method on the macro scale—

TABLE III

Calcium taken as mg of CaO	PERMANGANATE REQUIRED		Milligrams of CaO found
	Actual	Less blank	
0	0.24	0	
0.33	1.34	1.10	0.31
0.67	2.49	2.25	0.63
1.00	3.75	3.51	0.98
And in another series			
0	0.14	0	0
0.33	1.27	1.23	0.34
0.67	2.36	2.22	0.62
1.00	3.67	3.53	0.99

The presence of phosphates seemed to affect the size of the blank, as is shown in the following experiments (*vide* Table IV), in which varying amounts of potassium phosphate solution containing 1 mg of  $P_2O_5$  per c c were added —

TABLE IV

AMOUNTS TAKEN OF		PERMANGANATE REQUIRED		Milligrams of CaO found
Calcium mg	Phosphate mg	Actual	Less blank	
0·67	0	3·14	2·09	0·59
0·67	1	3·20	2·15	0·60
0·67	3	3·07	2·02	0·57
0	3	1·05	0	

The following controls of the method (*vide* Table V) were made using stones in which the calcium in the ash had been determined on a macro scale. —

TABLE V

Stone number	PERCENTAGE OF CaO FOUND		Difference
	Macro	Micro	
130	32·7	32·2	—0·5
39	21	12	—0·9
82	7·4	8·5	+1·1
79	17·9	16·1	—1·8
157	13·3	12·5	—0·8
28	0·2	0·4	+0·2

#### *Magnesium determination*

To the filtrate and washings from the calcium oxalate precipitate 10 drops of a solution of potassium phosphate ( $K_2HPO_4$ , 20 grammes per litre) are added



and 1 c.c. of strong ammonia, and the whole warmed in a water-bath and then allowed to cool and stand 24 hours. The precipitate of magnesium ammonium phosphate—if any—is then filtered off through a No. 44 Whatmann paper, washed with about 10 c.c. of ammonia-water, dissolved in normal sulphuric acid and made up to 24 c.c., correct to within 0.5 c.c., with normal sulphuric acid. To this are added—as for the phosphate estimation—20 c.c. of ammonium molybdate solution, 5 c.c. of stannous chloride and the whole made up to 50 c.c. with normal sulphuric acid. The colour is then read against a standard as in the phosphate determination. To provide a blank, the filtrate and washings of the blank calcium determination are used (*vide* *s*) and the value found for this blank deducted from the other results.

We found that the results of this micro method on stones in which the magnesium had been determined by a macro method came out consistently low, unless an allowance was made for the solubility of the magnesium ammonium phosphate in the ammonia water. The necessary correction is 0.012 mg. of  $P_2O_5$  per c.c. of wash water. Making this correction, the results of control estimations came very close to the results by the macro method (*vide* Table VI) —

TABLE VI

Stone number	MgO PER CENT FOUND		Difference
	Macro	Micro	
31	0	0	0
30	0	0	0
177	5.9	5.6	—0.3
191	6.7	6.9	+0.2
189	8.2	8.3	+0.1
138	10.5	9.6	—0.9
183	29.0	28.7	—0.3

#### *Oxalate determination*

The determination of oxalates in stones was unsatisfactory but of the various methods tried, the one described below gave the best results —

Ten to twenty milligrams of the stone powder are accurately weighed into a 15 c.c. centrifuge tube and heated for an hour with ten c.c. of normal sulphuric acid in a water-bath. The tube is then allowed to cool and stand 24 hours. The tube is then spun in the centrifuge and the clear supernatant liquid carefully pipetted off leaving only a small fraction of a c.c. Again 10 c.c. of normal sulphuric acid are added and the contents allowed to stand another 24 hours,

without heating, with occasional shaking. The centrifuging is repeated and the clear solution pipetted off and added to the previous extract. The combined extracts are then made up to 50 c c with normal sulphuric acid. The oxalates in this solution can then be estimated either by direct titration with N/100 permanganate or by precipitation as calcium oxalate, before titration. In the former case 10 c c are taken and titrated and an allowance made for the uric acid present if the stone contains this substance. The allowance made was 0.22 c c of permanganate and this figure was arrived at by treating pure uric acid with sulphuric acid as for a stone powder and titrating 10 c c of the resulting solution. In the latter case 10 c c of the solution are taken, 10 drops of 10 per cent calcium chloride solution added and the solution neutralized to methyl red as for the calcium determination. The precipitate of calcium oxalate is allowed 24 hours to form and then the determination proceeded with exactly as for the calcium determination (*q v*).

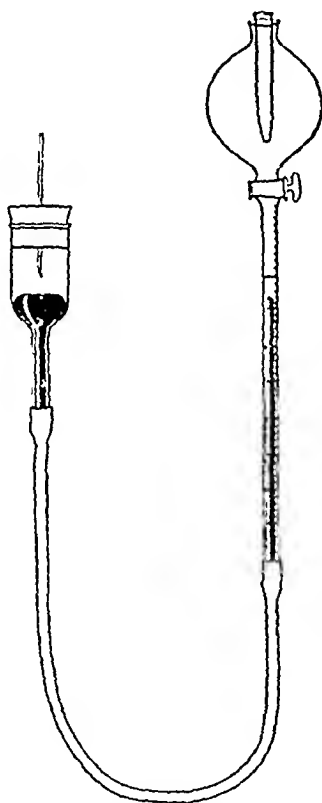
The results with stones in which the amount of oxalate had been estimated by a macro method are shown in Table VII —

TABLE VII

Stone number	PERCENTAGE OF $C_2O_3$ FOUND		Method
	Macro	Micro	
145	12.5	9.6	Direct titration
111	33.6	28.2	Precipitation
		26.7	Direct titration
52	6.8	4.7	Do
		5.5	Do
		5.7	Do
		7.8	Precipitation
		6.1	Do
126	Trace	1.5	Direct titration
75	2.0	1.6	Do
130	42.7	41.4	Do
39	2.5	2.4	Do
82	5.1	8.7	Do
79	19.0	20.4	Do
157	Trace	0	Do
28	0	2.8	Do

*Carbonate Determination*

A fresh portion of the dry stone powder is first tested qualitatively for carbonates. This can be done with a very minute amount of the stone, by putting a speck in a drop of water on a microscope slide, covering it with a cover-glass, and running a drop of hydrochloric acid under the cover-glass. The particles of stone are watched through the microscope and the appearance of bubbles indicates the presence of carbonates. If carbonates are found, the amount is estimated by liberating the carbon dioxide and measuring the volume of it, in the apparatus shown in the Text-figure. The apparatus can be constructed out



Text Figure

of materials to be found in most laboratories and consists of thistle or separating funnel with a good tap, graduated on the stem below the tap into c c and  $\frac{1}{10}$ ths. The lower end of this funnel is connected by a piece of rubber tubing to a mercury reservoir, which can be raised and lowered. The inside diameter of the stem of the funnel should be about  $5\frac{1}{2}$  millimetres giving about 42 millimetres length of stem for one c c. The stem should be about 25 centimetres long below the tap so that it will contain a volume of 5 c c or so. Sufficient mercury is put in the apparatus to still leave some in the reservoir when this is raised till the mercury rises above the tap. To use the apparatus the level of the mercury

is adjusted with the tap open so that it lies a few millimetres below the tap. About 10 milligrams of the powdered stone, accurately weighed, are then dropped in so that as much as possible falls through the hole in the tap into the stem. Any of the powder which sticks to the sides is then washed in with water dropped from a pipette, using as little as possible and not more than half a c.c. The process can be assisted by very gently raising and lowering the reservoir a millimetre or so. When all the stone has been washed into the stem, a few drops of water are added to fill the bore of the tap and the tap is closed. The mercury reservoir is then lowered. A little strong hydrochloric acid, containing a trace of powdered glass, is put in the bulb of the funnel, the tap cautiously opened, about  $1/10$ th to  $1/5$ th of a c.c. allowed to run into the stem, and the tap shut quickly. As the carbon dioxide is liberated, the height of the reservoir is adjusted so that the gas in the stem is kept roughly at atmospheric pressure until bubbles have ceased rising. The liquid in the stem is now super-saturated with gas and the disengagement of this is assisted by quickly raising and lowering the reservoir so that the mercury makes rapid excursions of equal distances on each side of the position it is finally going to take up. This should be continued for about half a minute and then the level of the mercury in the reservoir adjusted so that the gas is at atmospheric pressure, and a reading taken of both the volume of the gas and the volume of the gas plus acid. The shaking process should then be repeated until no further alteration takes place in the volume of the gas, and final readings taken. The height of the barometer and the temperature of the room are noted. To the volume of the carbon dioxide gas is then added an allowance of 0.85 c.c. per c.c. of acid for the gas dissolved in the acid. From the total volume of gas thus found, the weight of carbon dioxide is then calculated from one of the various tables published (4, 5) for weights of volumes of carbon dioxide, or by calculation\*.

As an example to make the calculation clear, suppose that 9.2 mg. of stone were taken and that the final readings of the volume of gas and liquid were 1.32 c.c. and 0.51 c.c. respectively. Suppose the barometer stood at 740 mm. and the temperature was  $17^{\circ}\text{C}$ . Allowing for the gas in the acid at the rate of 0.85 c.c. for 1 c.c. of acid, 0.51 c.c. of acid contain  $0.51 \times 0.85 = 0.43$  c.c. of gas so that the total volume of gas is  $1.32 + 0.43 = 1.75$ . For a pressure of 740 mm. and a temperature of  $17^{\circ}\text{C}$ , the weight of  $\text{CO}_2$  in one c.c. of saturated gas is 1.768 mg. (from the tables or by calculation). The weight of 1.75 c.c. is, therefore,  $1.75 \times 1.768 = 3.09$  mg. and the percentage of  $\text{CO}_2$  in the stone  $\frac{3.09 \times 100}{9.2} = 33.6$ .

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\* If  $p$  is the barometric pressure in millimetres,  $t$  the temperature Centigrade and  $p^0$  the vapour tension of water at  $t^0$  then the weight of  $\text{CO}_2$  in 1 c.c. of gas saturated with water is  $0.7056 \times \frac{(p - p^0)}{273 + t}$  milligrams.

In adjusting the level of the mercury reservoir before the readings, it is convenient to put it higher than the top of the mercury column in the stem by  $1/13$ th of the length of the column of acid, so that no correction is needed for the weight of the column of acid

A number of experiments were made using lithium carbonate in the above apparatus to determine the allowance to be made for the gas dissolved in the acid. It was found that, making an allowance of 0.85 cc of gas per cc of acid, the total gas obtained agreed with the theoretical quantity to within an average 0.1 cc, and always to within 0.2 cc and that the differences in different determinations were as often positive as negative. The method is, therefore, to be trusted to within say 0.2 cc of gas. The exact strength of the acid did not appear to affect the result within this error. Further controls were made using a sample of impure calcium carbonate and three vesical calculi in which the carbon dioxide had been determined on a macro scale with Schrotter's apparatus (*vide* Table VIII) —

TABLE VIII

Substance	PERCENTAGE OF CARBON DIOXIDE FOUND		Difference
	Macro	Micro	
CaCO <sub>3</sub>	40.0	40.5	+0.5
Stone	39.2	41.4	+1.9
Stone	38.0	38.7	+0.7
Stone	38.5	38.6	+0.1

## QUALITATIVE TESTS

For the detection of cholesterol some of the powdered stone is extracted with chloroform and the extract filtered through a small dry paper. The extract is reduced in bulk by evaporation or evaporated completely and then taken up in a little chloroform. To this solution are added with shaking —

- 1 Strong sulphuric acid—a red colour in the chloroform layer and a green fluorescence is the sulphuric acid and indicates cholesterol (Salkowski's test)

- 2 Ten drops acetic anhydride and two drops strong sulphuric acid—a blue or green colour indicates cholesterol (Liebermann = Burchard's test)

The former test can be made more delicate by using chloroform which does not contain any alcohol. Ordinary chloroform contains 1 to 2 per cent of alcohol and this can be got rid of by shaking the chloroform with an equal volume of water in a separating funnel, running off the chloroform layer and repeating the process. Six extractions are ample to get rid of all the alcohol. The chloroform is finally filtered through a dry paper to clear it from small drops of water. This purified chloroform, although it has a certain amount of water dissolved in it, gives a red colour in greater dilutions than ordinary chloroform. Chloroform without alcohol will not keep and has to be prepared fresh every few days. With washed chloroform 0.1 mg of cholesterol in 1 cc of chloroform can be detected by a marked green fluorescence in the sulphuric acid and a very faint red colour developing slowly in the chloroform layer.

The second test (Liebermann-Burchard's) is more delicate and will detect 0.02 mg of cholesterol in 1 cc of chloroform. The washing of the alcohol out of ordinary chloroform is unnecessary with this test.

As a qualitative test for uric acid and urates, the murexide test is recommended. A little of the powdered stone is evaporated to dryness with a few drops of strong nitric acid in a piece of porcelain dish on a water bath. If uric acid is present, a reddish residue remains which turns reddish-violet on the addition of a drop of dilute ammonia and blue-violet on the addition of a drop of caustic soda. Most of the nitrogen in stones is in the form of uric acid and when the total nitrogen is high, the presence of this substance can be presumed safely. Many stones, however, which only contain a few per cents of total nitrogen, do not give the murexide test and in these the nitrogen is presumably in some other form. In these stones the murexide test should always be done.

For the detection of creatinine a little of the stone powder is boiled for a few minutes with normal hydrochloric acid cooled, filtered, and to it added, an equal volume of saturated picric acid solution and drop by drop sodium hydroxide solution, until a deep orange colour indicates the presence of creatinine. Excess of sodium hydroxide destroys the colour (Jaffe's test).

If it is wished to extend this test to detect creatine as well as creatinine, the stone powder should be evaporated to dryness with the hydrochloric acid with the addition of a pinch of powdered lead. This converts creatine into creatinine and the test is then proceeded with as before.

Cystine can be detected by boiling a little of the stone powder with strong caustic soda for a few minutes and adding a drop of lead acetate solution. A black colour indicates the presence of cystine. It is a rare constituent of stones and we have never detected it in any of the stones we have examined,

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# THE VALUE OF A PROVOCATIVE DOSE OF PENTAVALENT ANTIMONY IN THE DIAGNOSIS OF KALA-AZAR

BY

L. EVERARD NAPIER, M R C S, L R C P (Lond ),  
*Kala-azar Research Worker,*

AND

C R DAS GUPTA, M B (Cal ),  
*Aucillary Kala-azar Enquiry, under Indian Research Fund Association,  
Calcutta School of Tropical Medicine and Hygiene*

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It was observed by Lieut-Col R N Chopra in the Pharmacological Department of the Calcutta School of Tropical Medicine and Hygiene that when antimony preparations were injected into the vein in an animal there was a considerable increase in the size of the spleen and that the normal rhythmical contractions were considerably increased. This effect was far more noticeable when the therapeutically more active pentavalent compounds were injected than when sodium antimony tartrate was given. Lieut-Col Chopra suggested that by this means the spleen was flushed out, and that this flushing might be an important step in the curative process. It occurred to the senior writer that advantage might be taken of this flushing action to increase the number of parasitized cells in the peripheral blood of kala-azar patients, and early in 1927 he and Dr R O A Smith took advantage of this observation in their sandfly-feeding experiments, placing the flies to feed on the patients immediately after an injection had been given. About this time the senior writer went on leave to Europe and Lieut-Col Chopra suggested to the junior writer that they should undertake a series of experiments to see if this increase actually occurred. A series of observations were made, the results obtained were very suggestive, but for one or two reasons they were not entirely conclusive. A short note reporting these preliminary observations was published in 1928 (Chopra and Das Gupta).

In these preliminary experiments only a very small number, 13, of definitely diagnosed cases of kala-azar were used, the observations were qualitative rather

than quantitative, and, as all cases in which the parasite was discovered in the peripheral blood before the antimony injection were excluded, a true comparison between the findings at the first, second, third and fourth examinations could not be made. As in nine out of the thirteen cases only one parasite was noted, one would be justified in assuming that in these cases a parasite was found because the examination was repeated four times, and not because there were more parasites present in the blood at the time of the subsequent examination. The suggestive points about these experiments were that in the other four cases more than one parasite was found, and that 13 parasites were found at the second examination, that is of blood taken after an interval of 10 minutes, against only 3 parasites at the fourth examination, that is of blood taken half an hour after the injection. After this interval the effect had obviously worn off.

In these circumstances we felt that it would be worth repeating these experiments, using a larger number of cases and making a quantitative estimate of the number of parasites, both before and at various intervals after the injection.

#### DETAILS OF THE PRESENT INVESTIGATION

*The patients*—Fifty kala-azar patients in whom a definite diagnosis had been made by demonstration of the parasite but who had previously received no treatment for kala-azar were used in this investigation. They were all in-patients in the Carmichael Hospital for Tropical Diseases under the charge of the senior writer, and the great majority of them were Indian males.

*The antimony compound*—Neostibosan (No 693b), which is the diethylamine salt of para-amino-phenyl stibinic acid, was the antimony compound used, primarily because it is the compound which is used for the treatment of all kala-azar patients in the above-mentioned hospital. The drug was given intravenously, 0.2 gramme in 4 c.c. of distilled water.

*The blood films*—Blood was taken in the usual manner from a finger prick and 5 or 6 ordinary thin blood films<sup>\*</sup> made on each occasion. The blood was taken just before, and 5 minutes, 10 minutes, 20 minutes and 30 minutes after the injection had been given. The films were stained by Leishman's method.

*The parasite counts*—The films were examined microscopically with a 1/12 in oil-immersion lens and a No 2 eye-piece. The leucocyte edges of the films of each group were examined until 500 leucocytes had been counted, the number of parasites and the number of parasitized cells encountered during this examination was noted. In the case of 25 of the patients no parasites were found during any of the 5 examinations, the findings in the remaining 25 instances are given in the Table. All the counts were made by the same observer, the junior writer.

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\*The thick-film method was used at first, but it was found that, although the majority of the leucocytes stained well, there were always a few badly stained ones in which there was an element of doubt as to the presence or otherwise of parasites, the counts were thus vitiated. These results were all discarded, and the thin-film method only was used in the observations reported.

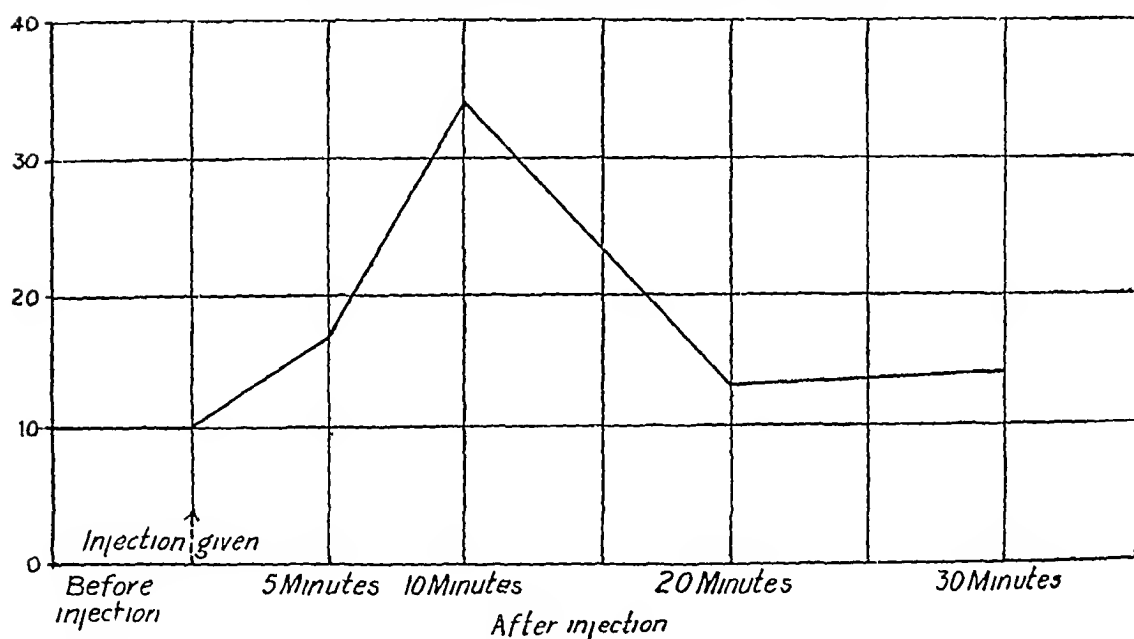
TABLE

Serial number	BEFORE INJECTION		5 MINUTES AFTER INJECTION		10 MINUTES AFTER INJECTION		20 MINUTES AFTER INJECTION		30 MINUTES AFTER INJECTION	
	Parasites	Parasitized cells	Parasites	Parasitized cells	Parasites	Parasitized cells	Parasites	Parasitized cells	Parasites	Parasitized cells
1					1	1	1	1	1	1
3					1	1				
4										
5			2	2						
6			1	1	4	4			3	3
9	1	1	1	1	1	1				
12			1	1	2	2				
13										
14	2	2			1	1				
17			1	1	2	2				
18					1	1				
23					1	1				
25					2	2				
26	6	3	3	2	1	1	2	2	2	2
27			1	1	2	2			1	1
32			1	1	1	1			1	1
33			1	1	1	1			2	2
36			1	1	1	1				
37	1	1	2	2	4	3	2	2	10	2
39	2	2			3	3	1	1	1	1
41					2	2	2	2		
42					1	1	1	1	1	1
44					1	1	4	3	1	1
49			3	2	1	1	1	1	1	1
50	1	1	1	1	2	2	1	1	1	1
	13	10	19	17	46	34	14	13	24	14
Total number of parasites and parasitized cells										
Total number of cases in which parasites were found	6		13		20		8		9	

## DISCUSSION ON THE RESULTS

Out of the 50 patients the parasite was only found in 6 (or 12 per cent) by the ordinary thin-film method. This is in keeping with previous observations. Knowles, Napier and Das Gupta (1923) found parasites in the peripheral blood of 19 per cent of 140 patients by examining an average of 32 films per patient. In every instance in which parasites were found before the injection, they were also found after the injection. Five minutes after the injection parasites were found in 13 cases (or 26 per cent), and after an interval of 10 minutes they were found in 20 cases (or 40 per cent). After 20 minutes and 30 minutes the positive cases had again fallen to 8 and 9 (16 and 18 per cent), respectively.

The number of parasitized cells found rose from 10 prior to the injection to 17 and 34, five and ten minutes after the injections and then fell again to

Number of parasitized cells in  $500 \times 50$  leucocytes

13 and 14, twenty and thirty minutes after the injection. A graph has been drawn showing the rise and fall in the number of parasitized cells found at different time intervals.

It is thus clear that there is a marked increase in the number of parasites present in the peripheral blood at intervals of 5 and 10 minutes after the injection, but neither the difference in the number of positive cases nor the difference in the number of parasitized cells between the 1st and the 4th and 5th examinations is sufficiently large to justify drawing the same conclusions regarding the examinations after the two longer intervals.

## CONCLUSIONS

The intravenous injection of an ordinary therapeutic dose of neostibosan has the effect of increasing the number of leishmania parasites in the blood of a

kala-azar patient. The greatest number of parasites will be found between 5 and 10 minutes after the injection.

In this series the number of parasitized cells found in smears taken 10 minutes after an injection had been given was 34, compared to 10 parasitized cells in the same amount of blood taken from the same patients prior to the injection. This increase is so marked that it would be worth while adopting the procedure described to facilitate diagnosis in cases in which the more certain methods of spleen or liver puncture, or blood culture cannot for any reason be employed. The interval between the giving of the injection and the taking of the blood for examination should be 10 minutes. Although in the experiments reported above neostiboson was used, there is no reason to suppose that any therapeutically active pentavalent compound of antimony would not act in a similar way.

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# ON AN IMPROVED METHOD OF DISSECTING SAND FLIES FOR PARASITOLOGICAL OBSERVATIONS

BY

S. MUKERJI, M.Sc.,

*Entomologist, Auxiliary Kala-azar Research*

*(Working under the Indian Research Fund Association)*

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IN parasitological investigations, especially, of the gut-contents of blood-sucking arthropods, the value of successful dissections of the alimentary canal cannot be overestimated. According to some authorities, dissections are superior in certain respects to microtome sections, in that, it not only shows the parasites *in vivo*, but also the exact position of the parasites in a particular organ and their tendency to migration, if any, to different regions.

The author, while carrying on dissections of infected and non-infected *Phlebotomus argentipes* Ann and Brun, struck upon a method which is simple in technique, effective, and needs but little practice on the part of the investigator. Patton and Cragg's (1913) method of dissection, though a simple one, has been found to be successful only in about 40 per cent cases. The main defect in this method lies in that the stomach, which is the widest portion in an insect gut, is to be pulled through a long distance if it is to come out through the posterior end. The four Malpighian tubules as well as the long diverticulum, which runs parallel to the alimentary canal, take a wrong and unnatural direction during this procedure, so that, there is every possibility of rupturing or snapping of the gut by these combined resistances. Further, the excision of the anterior end of the proventriculus invariably leads to the emergence of the parasites through the cut-end, and thus, in certain cases, is not desirable. Thus, the most natural way of dissection would be to allow the alimentary canal with the respective appendages, to remain in the most natural position during the process of pulling.

The following method was found by the author to be effective for specimens freshly killed or dead within 12 hours of dissection. The specimen or specimens to be dissected should be put as usual in a test tube partly filled with sterile normal saline solution and shaken gently for a few seconds to ensure the penetration of the fluid. The flies should then be allowed to remain in the tube for about 20 minutes

The insects, after this, should be taken out individually and placed on their edge on a clean slide with a drop of saline solution. Care should be taken to allow the drop of fluid to spread evenly,\* otherwise, surface tension will set in and hamper further progress. The next procedure is to cut off the wings, legs and other unnecessary appendages along with the two terminal segments bearing the external genital appendages with a lancet-shaped needle. If carefully done, it will be found that a considerable portion of the distal region of the alimentary canal will tend to come out through the dissected anal end. The anal region of the alimentary canal will thus be free. Now, the thoracic region of the fly should be held at the coxal ends of the legs with a fine but blunt needle† and the border of the head behind the eyes gently teased with a lancet-shaped needle with a motion that will tend to separate the head from the rest of the body. A careful and steady teasing will separate the head showing clearly the proventriculus, the paired globular salivary glands at the distal margin of the head, as well as a portion of the oesophageal diverticulum running parallel to the proventriculus. At this stage the excess fluid should be soaked up by means of a blotting paper rolled in the shape of a thin-pointed pencil. The dissected portion of the gut will now be found to lie flat on the slide on only a thin film of liquid. For a successful dissection, this is particularly important at this stage and the insect should not be allowed to float on excess fluid. Experience only will teach how much fluid is to be drawn off. It will be found that at this stage, the surface tension begins to act on the head and the dissected portion of the alimentary canal and keeps them fixed to a certain extent on the slide, thus, eliminating altogether the necessity of a second needle for holding the head. Successive short and steady pulls, directed towards the abdominal end, by holding the lateral edge of the thorax with the looped needle previously described will, in most cases,‡ allow the emergence of the entire alimentary canal. The entire organ along with its appendages, owing to its passage through a common lumen at the anterior end of the thorax, will lie more or less straight on the slide. Slight manipulations of the abdomen is necessary to get the appendages, especially, the 4 Malpighian tubules in position. The organ should now be re-floated in a drop of normal saline solution§ and examined under microscope for parasites.

The main principle of this method of dissection is to allow the surface tension to act on the parts to be dissected and examined, and the success depends

\* This is not difficult to attain, as a scrupulously clean slide will automatically prevent the formation of drops.

† This is easily made by making a small loop at the end of a fine needle.

‡ The dissection of a gravid female is slightly more difficult, since the ova inside not only cause a distention of the walls of the abdomen but also exert a certain amount of pressure on the distal region of the alimentary canal, which, in certain cases, is so strong as to cause a rupture during teasing and pulling.

§ Great contraction of the alimentary canal takes place after the addition of the saline solution. The organ should then be readjusted with a needle after soaking up the excess fluid with a piece of blotting paper. This contraction might be obviated by using Ringer's or Pictet's solutions as dissecting medium as suggested by Imms (1929).



on the adjustment of the required amount of moisture to allow of the proper action of the surface tension on the organ

The author is deeply indebted to his Chief, Dr L. E. Napier, Officer-in charge, Ancillary Kala-azar Research Calcutta School of Tropical Medicine and Hygiene, for his valuable criticisms on the subject

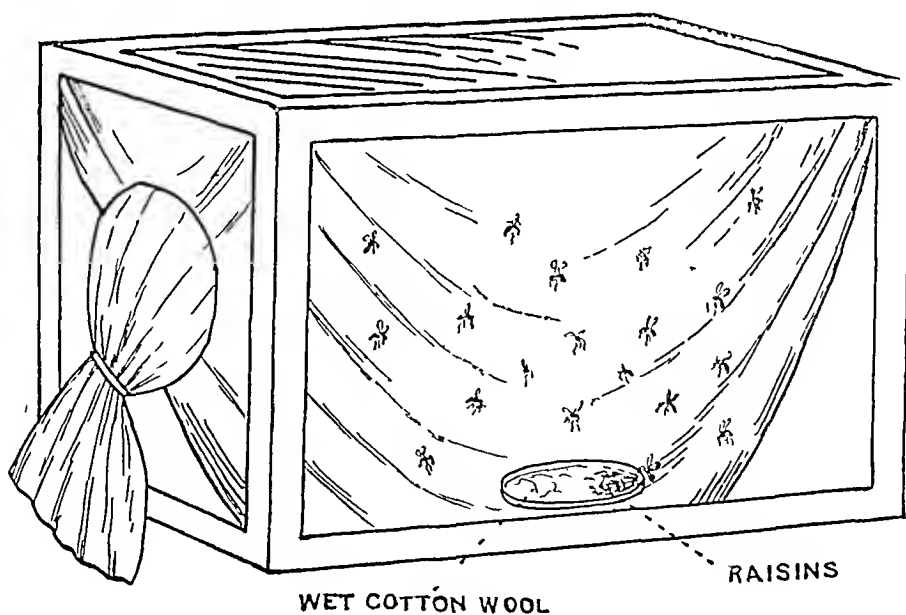
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the hours 11 p m and midnight The cases on which these mosquitoes were fed were such that they showed about 20 microfilariae per 20 cubic millimetre of peripheral blood at night A microfilaria count of the case was made on the night prior to the feeding and another at the time the mosquitoes were being fed on the patient A record was maintained of the details of the feeding experiments, the date of feed, name of patient, microfilaria count and the number of mosquitoes fed

Not all the mosquitoes in the feeding cage took the feed Usually some did not feed at all, while some had only a partial feed On the following morning, the unfed and half-fed mosquitoes were removed from the cage and only the full fed ones kept for further observation After the infective feed on the filarial case, the mosquitoes were not given any subsequent blood feed, but were kept on raisins and water

The feeding cage employed is of the ordinary type, slightly modified (*see* Text-figure) The cage consists of a wooden frame-work from which a muslin bag hangs on the inside, the top consists of a sheet of glass to facilitate easy examination of the insects The front side of the muslin bag comes off into a



Text-figure

sleeve, the mouth of which is fitted with an elastic band to ensure automatic closing of the sleeve The sleeve serves for letting in and taking out mosquitoes and also for thrusting in the hand of the filarial case for feeding the mosquitoes On the glass top, the details of the experiment are written A small petri dish with a few washed raisins and a pellet of cotton-wool soaked in water is kept in the cage and this dish is changed every day The pellet of wet cotton-wool to supply water to the mosquitoes is preferable to keeping water in a dish, since it is frequently found that mosquitoes get stuck in the open water in the dish and perish

The infectivity and the development of the embryos in the mosquitoes were studied both by dissections and by the cutting of serial sections. For the latter procedure, the specimens were fixed in warm Bles fluid and imbedded by the celloidin paraffin double-imbedding method. The sections were cut to a thickness of  $5\ \mu$ . This method of studying the embryos has been found to be a very reliable method as every part of the mosquito tissue is available for careful examination and there is no chance of missing any of the embryos in the mosquito.

#### THE DIFFERENT SEASONS

These experiments on the seasonal infectivity of *Culex fatigans* were carried out in Calcutta and it is necessary therefore to consider the weather conditions prevailing there during the different seasons of the year.

The daily maximum and minimum temperature and humidity records of Calcutta for the two years 1927 and 1928 are given in a statement at the end of this paper (Table I). Broadly speaking, there are three distinct seasons in Calcutta, the hot season, the monsoon or wet season and the cold season. The *hot season* starts in April and extends to June and during this period the average maximum daily temperature is above  $92^{\circ}\text{F}$ , and the minimum is between  $77^{\circ}$  and  $80^{\circ}\text{F}$ . A small amount of rainfall occurs during this season. The daily minimum relative humidity is generally low, frequently falling as low as 40 per cent. The *wet season* follows the hot season and covers the period July to September. During this season, there is a very heavy rainfall and the daily temperature does not exhibit any large range between the maximum and the minimum for the day. During this period, the temperature ranges usually between  $79^{\circ}$  and  $89^{\circ}\text{F}$ . Humidity is invariably high, rising as high as 95 per cent or more during the mornings and falling to 80 per cent in the afternoons. The average minimum humidity during this period is 84 per cent. The *cold season* starts about the middle of November and ends in February. During this period, the atmospheric temperature is low, the average daily maximum temperature is between  $76^{\circ}$  and  $83^{\circ}\text{F}$ , and the minimum, between  $57^{\circ}$  and  $62^{\circ}\text{F}$ . The minimum daily humidity is low, usually below 50 per cent. October and part of November form a transition period between the monsoon season and the cold season, and similarly, the month of March links up the cold season with the hot season.

#### SEASONAL INFECTIVITY

*Wet season.* A series of experiments were conducted during the months July, August and September, the monsoon season, when the atmospheric temperature is neither too high nor too low and the humidity is very high. The temperature is usually between  $79^{\circ}$  and  $89^{\circ}$  throughout the day and night. The humidity is always above 80 per cent. During these experiments in which more than 500 mosquitoes were dissected or sectioned serially, it was found that the infectivity rate was high, the number of embryos undergoing development in

each mosquito was also found to be high, i.e., usually more than 15 to 20. During this period, the time taken for the completion of the mosquito phase of the embryos was found to be quite low, i.e., 11 days in July and 10 days during August and September. The month of August was found to be most favourable for the embryos since a large number of them underwent development and the rate of growth was rapid.

In the month of October, a transition period between the monsoon months and the cold season, when the temperature rises slightly higher than during the monsoon season, and the humidity is somewhat lower, the time taken for the completion of the mosquito phase of the embryo was found to be 10 days as during September. In November just before the onset of the cold season, the temperature is much lower than in October or September and the minimum humidity falls very low, to as much as 50 per cent. It was then found that it took 15 days for the embryos to mature.

*Cold season* During the cold season December to February, it was found that the mortality among the mosquitoes was heavy even under laboratory conditions. During this period the infectivity of the mosquitoes was very low, and the number of embryos developing in each mosquito was very small, 1 to 4 being about the maximum found in a mosquito during this season. The embryos took a considerably longer time to mature and it was found that 18 to 20 days were usually required for the completion of the development even under favourable laboratory conditions. In a series of experiments carried out in February, it was found that only a few out of a large number of fed mosquitoes took the infection. Even among the few that took the infection, the number of embryos was hardly more than one or two.

During March, the temperature is higher than during the cold season, but the humidity falls as low as 30 per cent. During this month, there was heavy mortality among the mosquitoes. The infectivity rate was very low and the time taken for the embryos to mature was 16 days, which is slightly less than that during the cold season.

*Hot season* During the hot season, mortality among the mosquitoes was very heavy. The infectivity rate was fairly high, i.e., between 50 and 60 per cent, and the time taken for the embryos to mature was 11 to 12 days. The number of embryos was however small.

#### CONTROLLED HUMIDITY AND TEMPERATURE

It was then considered whether it was possible to obtain a heavier infection of the mosquitoes or a more rapid development of the embryos during adverse seasons by controlling the temperature and humidity conditions. With this object in view, comparative experiments were carried out in February 1928 to study the development of the embryos in the mosquito when kept at room temperature and when kept under conditions similar to those prevailing during the wet season. Out of a batch of mosquitoes fed on the same patient at the same time, one half

the number was kept at room temperature while the other half was kept in a wet incubator in which the temperature and humidity were controlled. In this wet incubator, the temperature was maintained constantly at 82°F and the humidity was kept at 90 per cent. The batch that was kept at room temperature was subject to the conditions of temperature and humidity prevailing at the time. The temperature ranged between 62°F minimum and 86°F maximum and the minimum humidity fell as low as 17 during some days, with an average daily minimum of 25 for the whole period.

In the two portions of the same batch fed at the same time on the same case, the results were very different. Among the mosquitoes kept at room temperature, most were negative while only one had one or two immature embryos. Among those mosquitoes that were kept in the wet incubator, every one had embryos and in many the embryos had matured and reached the free proboscis stage. In each of these mosquitoes 5 to 10 embryos were found. The time taken for the completion of the mosquito phase of the embryo also varied considerably. In the monsoon incubator, it took 12 days for the embryos to reach the mature stage. It was mentioned above that only one of the specimens kept at room temperature had any embryos. Even in this specimen, on the 15th day when it was killed, and subsequently sectioned, it was found that the embryos were still very immature. In these experiments, to avoid any possible omission during the examination of dissected material, all the mosquitoes of the two batches were serially sectioned.

In the above series of comparative experiments, the only difference between the two batches is the temperature and humidity conditions. The one batch was exposed to winter conditions in Calcutta, while the other was kept in a highly humid condition with a temperature constantly at 82°F. The difference in the behaviour of the embryos in the two batches is very interesting.

#### DISCUSSION OF RESULTS

These observations on the development of filaria embryos in *Culex fatigans* at different seasons of the year show how considerable is the influence of climatic conditions on the development of filaria embryos in the body of its intermediate host, the mosquito. During the monsoon months, a large proportion of the embryos ingested by the mosquito survive in the mosquito and complete their development. The time taken for the completion of the development is shorter during this season than at any other time of the year, namely, 10 days. During the cold season, the number of embryos that mature form only a very small proportion of the total number actually ingested by the mosquito. The time taken for the embryos to mature is greatly prolonged, namely, 18 to 20 days. During the hot season, the time taken for the embryos to mature is shorter than during the cold season (i.e., 11 to 12 days) but the number of embryos that mature is small compared to the total ingested. If during any of the adverse periods, the fed mosquitoes are kept under conditions of controlled temperature

and humidity, similar to that prevailing during the monsoon season, it is possible to produce a heavier infection and to accelerate the development of the embryos

Temperature and humidity are the two factors that bring about these different results in the experimental infection of *Culex fatigans*. Low temperatures below 75°F and high temperatures above 92°F seem to have an inhibitory effect on the development of filaria embryos in *Culex*. There appears to be an optimum temperature namely, between 85°F and 90°F for the most rapid development of the embryos. Humidity has an even greater influence on the development of the embryos. During periods of high humidity the percentage of infected mosquitoes is high and the intensity of infestation is heavy\*. During low humidity periods, both the infectivity as well as the infestation are very low.

The photomicrographs of the sections of infected mosquitoes illustrate this point. Plate LVI, fig 1, shows the heavy infestation of a *Culex* mosquito experimentally infected during the monsoon season. Here a large number of half mature embryos are seen imbedded in the thoracic muscles. Plate LVI, fig 2, is a transverse section through the thoracic muscles of an infected mosquito showing the very large number of embryos imbedded in a mosquito which was infected during the monsoon season. Plate LVII, fig 1, is a section through the head and thorax of *Culex* experimentally infected during the wet season. It shows the enormous number of mature embryos in the thorax, head and proboscis of the mosquito. Such heavy infestations are characteristic of the wet season and have not been observed during any other season. A combination of a constant optimum temperature with a very high atmospheric humidity such as prevail in Calcutta during the wet season, account for the high infection rate as well as the heavy infestation that occur among experimentally fed mosquitoes.

The mosquitoes that were kept in the wet incubator, although the infection rate was very high, did not give such a heavy infestation as that obtained during the wet season. The temperature in the wet incubator was maintained constantly at 82°F, while during the wet season, the prevailing temperature is usually at about 85°F reaching as high as 90°F. It is seen how even in the presence of a high humidity factor, a lowering of the temperature retards the development of the embryos and also reduces the intensity of infestation. While most of the embryos in the wet incubator series matured in 12 days, some took a considerably longer time. In the section, the photomicrograph of which is given in Plate LVII, fig 2, two mature embryos are seen in the head, while in the thorax, two immature ones are seen imbedded in the thoracic muscles. These latter embryos are at least three days behind the mature stage and this was the condition in a mosquito of the wet incubator series which was killed 15 days after the infective feed. While some of the embryos had matured in 12 days, others took a much

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\* By the term 'Infection rate,' we mean the percentage of mosquitoes found to have embryos in them. By the term 'Intensity of infestation,' we mean the number of filaria embryos found within a single mosquito.

longer time. The lowness of the temperature in the wet incubator, namely 82°F, appears to be the cause of the retarded development.

Although very little has been recorded so far on the influence of temperature and humidity on the development of *P. bancrofti* in *Culex fatigans*, some very remarkable work has been carried out on the relation of temperature and humidity to the infection of Anopheline mosquitoes with malaria parasites. It is of interest to consider the previous work on this question as it seems to throw light on the present observations on the development of filaria embryos in *Culex*.

Bentley (1911) in his classic work in Bombay first pointed out the importance of humidity and temperature on the infection of *Anopheles* with malaria parasites. He pointed out the definite relationship between the period of high atmospheric humidity and the heaviest infection of *Anopheles* during the three months July to September, a period of very high and uniform humidity. He further pointed out that when humidity was low there was no infection of the *Anopheles*, regardless of the temperature being high or low. He considered, therefore, that humidity was one of the most important factors in the production of the infection in the *Anopheles*. Gill (1921) is of opinion that humidity has no direct effect on the development of the malaria parasites in the mosquito and that the influence of relative humidity on the infection of *Anopheles* is solely by prolonging the life of the mosquito, long enough for the parasites to complete their development. Bruce Mayne (1928) working in Saharanpur found that although he dissected 2,000 specimens of *Anopheles culicifacies* no infected specimens were encountered till the 15th August when he found the first infected specimen and after that date, within 25 days found four more infected mosquitoes of the same species. His paper shows the correlation of high relative humidity and the appearance of infected mosquitoes.

Wenyon (1926) while discussing the question of the effect of humidity on the development of malaria parasites in the mosquito, says 'As regards the effect of humidity on the active development in mosquitoes, there is no evidence that this factor plays any part. Provided there is sufficient moisture in the air to enable the mosquitoes to live, the malaria parasites will develop normally. Temperature is a much more important factor than humidity. As Gill (1921) has pointed out, the spread of malaria may, however, be affected by lack of humidity, because the mosquitoes which hatch and ingest parasites may not live long enough for sporozoites to appear in the salivary glands.' Wenyon would not appear to have given due weight to the observations of Bentley in Bombay where he found that in the absence of proper humidity no infection happened, whatever the temperature may be.

The present observations show how temperature is a very important factor in the development of filaria embryos in *Culex fatigans*. But humidity has been found to have an even more important influence on the development of the embryos. In this matter, the observations of Bentley on the development of malaria parasites in *Anopheles* are illustrated in the present instance with regard to filaria embryos. A high humidity is necessary not only from the biological

aspect of prolonging the life of the mosquito to enable the completion of the development of the parasite, but also to facilitate the development of the embryos. In the absence of the proper humidity, the infection rate as well as the degree of infestation fall very low. During the hot season, with a high atmospheric temperature, but with a very low humidity, the degree of infestation and the percentage of infection are very low even when mosquitoes were kept alive for a sufficiently long time to enable the embryos to complete their development. The present authors consider that with regard to the development of filaria embryos in *Culex*, a high humidity is as important a factor as an optimum temperature, not only to enable the mosquitoes to live long enough to complete the development, but also in enabling a higher infection rate and a heavier infestation.

#### SUMMARY

While experimentally infecting *Culex fatigans* with embryos of *Wuchereria (Filaria) bancrofti* by feeding the mosquitoes on filarial cases, it was found that the percentage of infection and the intensity of infestation, as also the time taken for the embryos to complete their development, varied from season to season. Systematic work was carried out at Calcutta during different seasons of the year. It was found that the monsoon months when the humidity is high and the temperature optimum the heaviest, infection and a high degree of infestation occurred experimentally. During this season, the rate of growth of the embryos was most rapid, complete development taking place within 10 to 11 days. During the winter months, the infection rate was very low, infestation small and the time taken for the completion of the development much prolonged, i.e., 18 to 20 days. During the hot season, the period of development is not prolonged (11 to 12 days) but the degree of infection as also of the infestation is low.

If the temperature and humidity conditions are made favourable, it is possible to obtain rapid development even during adverse periods. This has been shown by the behaviour of two batches of mosquitoes fed on the same case during the month of February, one of which was kept at room temperature, while the other batch was kept in a wet incubator in which a high humidity and a moderate temperature was maintained. In the former, there was extremely slow development and the infection rate was very low and the number of embryos very small. In the latter the infection was heavy and the development was more rapid.

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- |                       |   |
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TABLE I

*Statement of meteorological records of Calcutta during 1927 and 1928*

Months	Daily maximum temperature (average)	Daily minimum temperature (average)	Daily maximum humidity (8 a m average)	Daily minimum humidity (4 p m average)	Rainfall monthly total
1927	°F	°F	Per cent	Per cent	Inches
January	77.6	58.5	93	48	0.4
February	82.3	62.0	85	39	1.2
March	91.2	67.4	81	30	0.2
April	97.3	77.3	86	46	2.1
May	94.9	77.6	88	57	4.9
June	92.0	80.2	92	75	11.5
July	89.1	79.5	93	79	8.5
August	88.9	79.0	95	81	7.0
September	90.0	78.6	95	80	7.0
October	89.7	75.3	94	60	2.9
November	83.7	65.1	90	47	0.2
December	79.0	57.8	93	45	0.0
1928					
January	79.4	58.2	87	43	0.2
February	85.9	62.2	80	34	0.0
March	96.1	70.7	76	28	0.1
April	96.0	75.7	80	44	3.2
May	94.5	78.4	84	64	7.1
June	89.6	78.6	90	81	18.0
July	88.8	79.0	92	81	22.1
August	89.5	79.0	91	82	16.3
September	91.0	79.5	93	78	8.7
October	88.5	77.0	93	72	2.9
November	84.7	66.5	89	41	0.0
December	79.5	58.6	88	45	0.0

#### EXPLANATION OF PLATE LVI

- Fig 1 Longitudinal section through thorax of a *Culex* infected during August, showing numerous embryos in the thoracic muscles
- „ 2 Transverse section through a mosquito infected during August showing the large number of embryos imbedded in the thoracic muscles The embryos are all seen in section

PLATE LVI

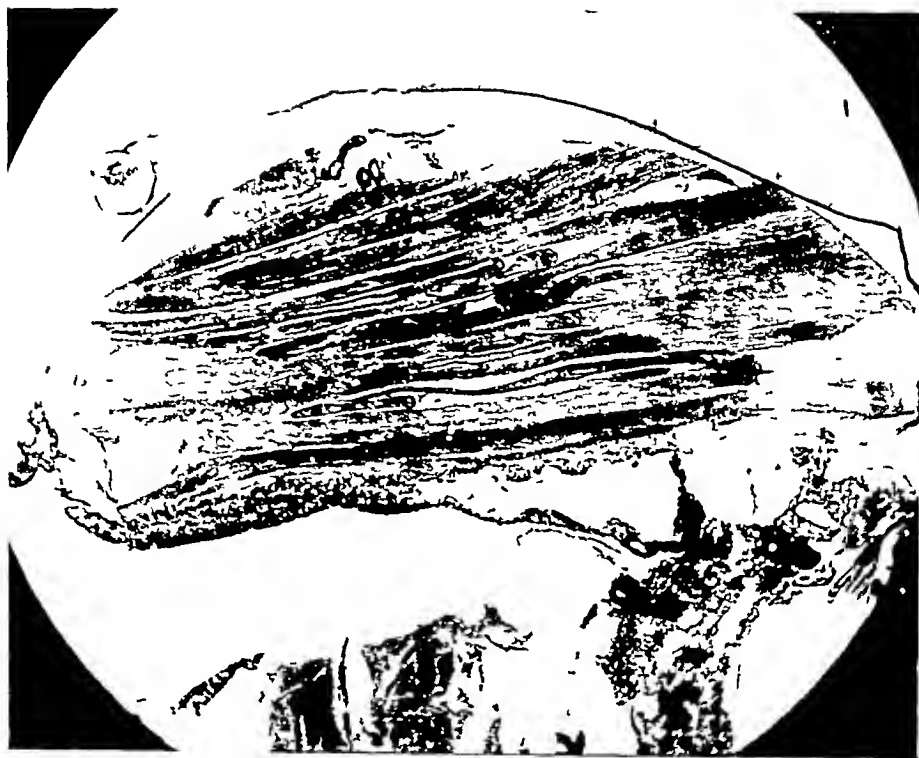


Fig 1

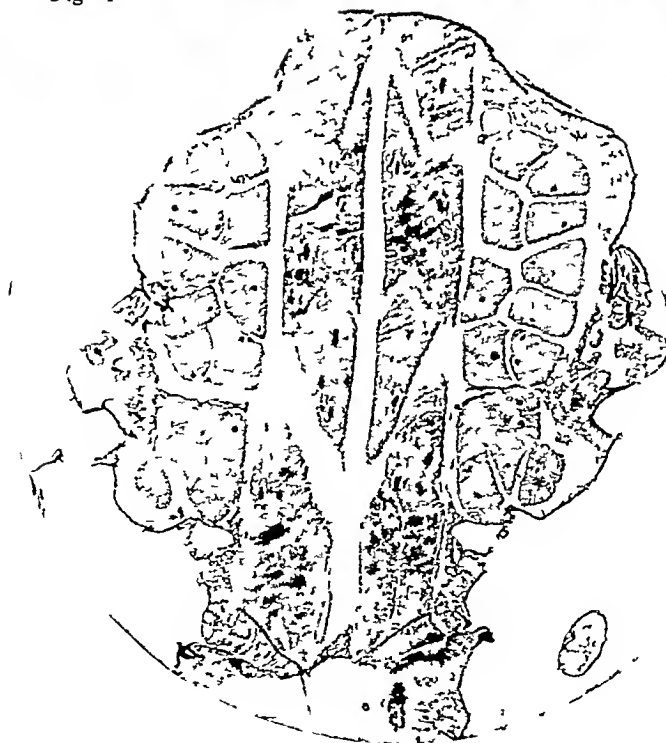


Fig 2

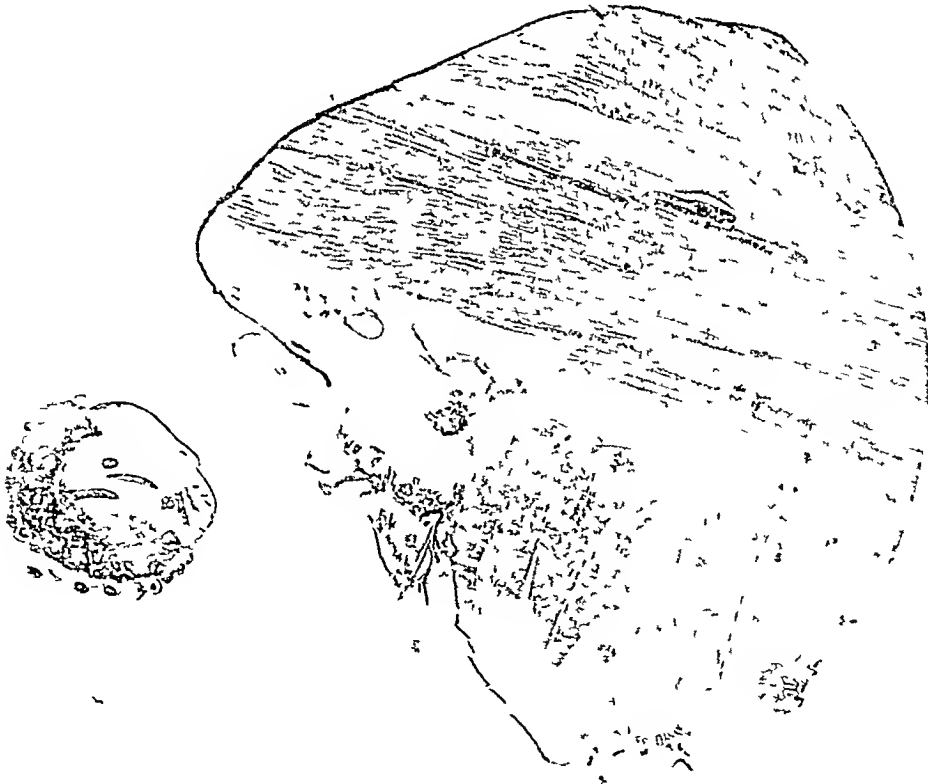
PLATE LVII



Fig 1



Fig 2



#### EXPLANATION OF PLATE LVII

- Fig 1 Section through head and thorax of a *Culex* infected during the wet season, showing numerous mature embryos in thorax, head and proboscis
- „ 2 Section through a *Culex* kept in wet incubator after an infective feed in February (killed nine days after feed), showing numerous embryos in thoracic muscles. Specimens from the same batch kept at room temperature did not develop any embryos at all
- „ 3 Section through head and thorax of a *Culex* fed in February and kept in a wet incubator. Killed 15 days after the infective feed. Two mature embryos are seen in section in the head and two immature ones are seen imbedded in the thoracic muscles



suggested are capable of producing a permanent cure in a large number of cases of chronic benign tertian malaria. The fact that some of these patients only received 4 days of plasmoquine treatment, suggests that shorter courses of plasmoquine, if followed by more prolonged quinine treatment, may also be an efficient method of treatment, or least deserves further trial.

*Series II* Amongst the 44 patients in this series relapses were detected in three cases but in two patients a full observation period of 8 weeks by blood examination could not be carried out, the observation periods being 2 and 5 weeks respectively. The later histories of these two cases were traced and in neither instance had relapse been reported at a later date.

From these figures it would seem that a high percentage of permanent cures can be obtained, with little toxic risk in a robust population, with even as small a daily dosage as 0.04 gram of plasmoquine, if this is combined with quinine. The percentage is so high that it seems to us doubtful whether a higher dosage is justifiable in an attempt to obtain a few extra cures, which could probably be obtained by another course of treatment if relapse occurs.

*Series III* One of the six patients treated by intramuscular injection was lost sight of before the end of the period of observation and in none of the other five was a relapse detected. The number of patients treated with this low dosage of plasmoquine are too few on which to generalize, but suggest that even smaller doses than 0.04 gram of plasmoquine, in combination with quinine, may produce a high percentage of permanent cures.

*Quinine Control Series* Among the 38 controls, relapses were observed in 15 or about 42 per cent.

#### THE EFFECTS OF TREATMENT ON THE DURATION OF *P. vivax* IN THE PERIPHERAL BLOOD

For the purposes of comparison the duration of parasites in the peripheral blood as determined by the thick-film method have been given in Table II, in which the results observed in our previous work (Sinton and Bird, 1928) have been included.

From this table it will be seen that in only two instances (3 per cent), out of the 71 patients observed, were parasites found after 36 hours from the commencement of treatment, when quinine was given in solution along with plasmoquine, while in previous work where the quinine was given in tablet form in the same doses and with larger doses of plasmoquine, 17 per cent of the cases still showed parasites after 48 hours. The quicker rate of disappearance in the former case may have been due to the better absorption of the quinine from the standard quinine solution than from the tablets.

From these results it would appear that the combination of plasmoquine and quinine has probably a more rapid action in clearing the peripheral blood of parasites than either drug given alone.

TABLE II

Series	TREATMENT			NUMBER OF CASES SHOWING PERCENTAGE AFTER HOURS —														
	Drugs	DATA DOSES		Total cases	0		24		36		48		72		96		120	
		Plasm	Quinine		Cases	Per cent	Cases	Per cent	Cases	Per cent	Cases	Per cent	Cases	Per cent	Cases	Per cent		
Grammes	Grains	Cases	Per cent	Cases	Per cent	Cases	Per cent	Cases	Per cent	Cases	Per cent	Cases	Per cent	Cases	Per cent			
PM *	Plasm	0.08	N/1	46	100	42	91.0	?	?	26	56.5	8	17.4	3	6.5	0	0	
PMC *	Pl Comp	0.10	20	34	100	18	53.0	?	?	6	17.0	1	3.0	0	0.0	0	0	
PMQ I	Pl and Q	0.06	20	17	100	8	47.0	0	0	0	0.0	0	0.0	0	0.0	0	0	
PMQ II	Pl and Q	0.04	20	48	100	13	27.1	2	4.1	0	0.0	0	0.0	0	0.0	0	0	
PMQ III	Pl and Q	0.03†	10	6	100	5	83.3	0	0	0	0.0	0	0.0	0	0.0	0	0	
Control	Quinine	N/1	30	36	100	18	50.0	4	11.1	1	3.7	0	0.0	0	0.0	0	0	

\* The plasmoquine and plasmoquine compound series already recorded (Sinton and Bird 1928)

† By intramuscular injection



## THE EFFECTS OF TREATMENT ON TEMPERATURE

The 'average duration of fever' in our previous series of patients treated with plasmoquine alone was 0.8 days, while in patients treated with plasmoquine compound the average was 0.3 days.

Among the 17 patients in Series I, the average duration of fever was 0.44 days and in no case did the fever last more than half a day. Of the 48 patients in Series II, the maximum duration of fever was  $1\frac{1}{2}$  days in one instance and the average duration 0.35 days. Among the few cases treated by intramuscular injections the average duration of fever was 0.75 days, while among the controls the average was 0.60 days. The latter figure is higher than the average of 0.31 days found in previous work with the cinchona alkaloids in 1,127 cases, but is probably accounted for by the fact that in the control series no alkali was given with the quinine, while in the previous work a large number of the patients received this adjuvant.

These figures help to confirm our previous opinion and that expressed by other workers, that the combination of quinine with plasmoquine produces a more rapid abolition of fever than the latter drug alone.

## OTHER EFFECTS OF TREATMENT

The spleen rate among the cases treated was about 25 per cent before the commencement of treatment, and in no cases was splenic enlargement detected after the completion of treatment. We did not find that the rate of decrease was any more rapid amongst the plasmoquine cases than among the controls.

Among the patients receiving 0.06 gm plasmoquine daily, only 44 per cent showed a gain in weight at the end of treatment and there was a total average loss of about  $\frac{3}{4}$  lb per patient. Among those receiving only 0.04 gm plasmoquine, 63 per cent gained in weight and there was a total average gain of 1 lb per patient.

Among the controls treated with 30 grains of quinine daily, 50 per cent gained weight with an average gain of  $\frac{1}{2}$  lb per patient. The gain is not so great as that recorded in our previous control cases (Sinton and Bird, 1928), which is probably accounted for by the fact that in this series of controls the dosage of quinine was greater over a much longer period.

The percentage of hæmoglobin were taken in 7 cases in Series I before and after treatment and an average gain of 9 per cent was recorded. A similar gain was found among 8 cases in the control series.

## DISCUSSION OF THE RESULTS OF TREATMENT

There is no doubt that, in plasmoquine, a very valuable drug has been discovered for the treatment of some forms of malaria, not only for the radical cure of benign tertian malaria, but possibly also for the quartan form. It also has a destructive action on the gametocytes of *P. falciparum*, on which quinine has little or no effect. Unfortunately, the optimum effective dosage and length

of treatment necessary to produce a permanent cure of the disease has not yet been settled in all its detail. The determination of the safe curative dosage is a matter of the utmost importance and until this is known the use of the drug should be considered to be still in the experimental stage.

*Dosage.* The original dosage used, when the drug was first introduced, was in some instances as much as 0.12 to 0.20 gm daily and some workers (Hasselmann and Hasselmann-Kahlert, 1928, Krauss, 1929) are apparently still using these or even larger doses although the makers and most other workers have reduced the maximum daily dosage to 0.06 gm. The results recorded in this paper and those of many other investigators, indicate that equally good results can be obtained with these smaller doses. This daily dosage, on account of the toxic symptoms developed by some of our patients, is in our opinion too large for general use, more especially if continuous treatment is given.

Some workers state that 0.06 gm daily is the maximum dosage which can be given without ill-effects (Muhlen, 1927, Schulemann and Memmi, 1927, Fischer, 1928, Brilmmer, 1928). Cordes (1928), however, who started with daily doses of 0.08 gm reduced his dosage to 0.06 gm and finally to 0.04 gm on account of ill-effects, and even then some of his patients developed severe toxic symptoms. Wallace (1928) found that a reduction of the dose from 0.06 to 0.04 gm daily when combined with quinine produced almost equally good results. MacPhail (1928) thinks that 0.04 gm of the drug given with quinine can safely be administered under very ordinary supervision while Fischer and Weise (1927) state that the maximum daily dose free from ill consequences is 0.03 gm.

Our experience has been that doses of 0.06 gm daily cause toxic symptoms even amongst robust patients, while with daily doses of 0.04 gm, which gave nearly equally good results in the production of a permanent cure in chronic benign tertian malaria when combined with quinine treatment, such symptoms as appeared were of a mild character. In view of the stalwart character of our patients we arrived at the conclusion that even 0.04 gm daily may be too large for general use, except under medical control. The experiments with 0.03 gm doses were too few from which to draw any general conclusions, but they indicate that further trials of this dosage in combination with quinine should be carried out. It is possible that such trials may show that either a longer duration of treatment, or larger doses of quinine, may be necessary to get an optimum result. Even if it is found that with such small doses of plasmoquine the relapse rate is somewhat higher, if it is less than 20 per cent it would justify this dosage, if toxic symptoms can thus be avoided.

*The combination of quinine with plasmoquine* is clearly indicated. It can be seen from our results that when these two drugs are combined, a higher percentage of radical cures is obtained by smaller doses than when either of the drugs is given separately in larger doses. It is also seen that plasmoquine alone is inferior to quinine in the control of fever in benign tertian malaria, indeed some authorities recommend that the fever in malaria should first be cut short by quinine treatment before plasmoquine is started. In addition it has been asserted

that quinine tends to reduce the toxicity of the plasmoquine, but we have obtained no definite confirmation of this in our work

The facts that the combination of these two drugs produces better results in the radical cure of the disease, the disappearance of parasites from the peripheral blood and the cure of symptoms, suggest that each drug may be an adjuvant to the other and that it is possible that plasmoquine given in smaller non-toxic doses, although it may not kill all the parasites, yet, may so damage them that quinine can complete the destruction. This destruction, as pointed out above, may possibly be increased by larger doses of quinine or a longer duration of treatment. It is therefore recommended that tests along these lines be carried out and also that the combination of quinine with alkali should be tested instead of quinine alone. The use of alkali might also tend to protect the liver, which seems to bear the brunt of the toxæmia.

The combination of plasmoquine and quinine, 'plasmoquine compound,' issued by the makers contains the proportions of 0.01 grm plasmoquine to 0.125 grm (2 grains) quinine in each tablet. When these are given for treatment, if the daily dosage of plasmoquine is reduced to 0.06 grm, the dosage of quinine is only 0.75 grm (12 grains) quinine, while with 0.04 grm plasmoquine only 0.5 grm (8 grains) quinine are taken. We consider the administration of quinine with plasmoquine in its present form of plasmoquine compound tablets unsuitable for general use for the following reasons — (a) If a mixed infection with *P. vivax* and *P. falciparum* is present, perhaps undetected, the amount of quinine with the smaller doses of plasmoquine is much too small for the treatment of the latter infection, or even to control malarial symptoms in some cases. (b) The dose of quinine is too small to be optimum for the reduction of fever and the quick control of the clinical symptoms of the disease, which is what the patient expects. (c) The use of quinine in solution gives a greater and more rapid absorption of the drug than when it is given in solid form. (d) The administration of tablets of quinine followed by quinine in solution is much cheaper than the use of tablets of plasmoquine compound. (e) The issue of two strengths of plasmoquine compound tablets has led to confusion in dosage. When the drugs are given separately, such confusion is unlikely to arise.

*Duration of treatment* The results obtained by Sinton and Bird (1928) indicate that a continuous course of treatment produces more permanent cures in chronic benign tertian malaria than does an interrupted course. If the results of Series PM I and PMC I, both interrupted treatments, be compared with the results of the present research, they go to show that with the continuous course of treatment almost equally good results in the production of a permanent cure can be obtained with a smaller daily, and a smaller total, dosage of plasmoquine as with the interrupted course and with less danger of toxæmia. The use of a continuous course is preferable for it is easier to get patients to carry out a treatment, if they know it will finish within a definite short period than to get them to take a treatment of longer duration, and in which they are liable to forget the dates they should attend for treatment. If a treatment is to extend

over a period of say 5 weeks patients are much more liable to be lost sight of before the treatment is completed, than when a course of 3 weeks or less is necessary. In the latter case one is in daily touch with the patients and can therefore see the progress of treatment and detect toxæmia at an early date, while if the treatment is interrupted the patient may not attend in the intervals, and delayed toxic symptoms may develop during these periods.

The minimum daily treatment in relation to dosage has not been determined. Our results would go to show that with a daily dose of 0.06 gm the maximum duration of treatment in chronic benign tertian malaria is 3 weeks, but the fact that many of the patients who received plasmoquine for shorter periods but who continued quinine did not relapse, suggest that probably with this daily dosage in combination with quinine a permanent cure would be obtained in a much shorter time, possibly even in 10 days, but that the chances of toxæmia with this dose are greater than with smaller doses.

Series II treated with a daily dosage of 0.04 gm of plasmoquine and 20 grains quinine for 21 days only showed an average relapse rate of about 8 per cent and was followed by few toxic symptoms in the robust population treated by us. The occurrence of even a few toxic cases amongst our population suggests that even although the duration of treatment may be correct, yet the dosage may be too high for general use. We therefore suggest that further experiments should be carried out with (a) 0.03 gm plasmoquine combined with 20 grains quinine daily for 3 weeks, (b) 0.03 gm plasmoquine daily for 14 days with 30 grains quinine daily for the first week and 20 grains daily for the second week, (c) 0.04 gm plasmoquine daily for 14 days combined with 20 grains of quinine, and (d) 0.03 gm plasmoquine with 20 grains of quinine for 14 days. Even if it is found that with the smaller doses of plasmoquine the relapse rate is somewhat higher, if it is less than 20 per cent, it would be justified if toxæmia was avoided.

#### 6. TOXIC MANIFESTATIONS DURING PLASMOQUINE TREATMENT

Most workers who have used plasmoquine in treatment have observed toxic symptoms of greater or less severity in some of their patients. Many observers seem to think that the appearance of toxic symptoms is of comparatively little importance, if treatment is stopped or the dosage of the drug reduced immediately they are detected, but, as will be seen in the section on 'The Use of Plasmoquine in Treatment outside Hospital Practice,' there are many medical men who do not agree with this view.

#### *Signs of plasmoquine toxæmia*

The commonest indications of commencing toxæmia with plasmoquine are cyanosis, or slight gastro intestinal disturbances, or the two conditions together. The rapidity with which these conditions disappear without leaving any apparent ill-effects in the majority of instances when treatment is stopped, make them

seem usually of trivial import, so some workers only cease treatment when more severe symptoms have arisen. We believe, however, with Schiassi and Merighi (1928), that the occurrence of even these slight toxic symptoms cannot be disregarded as evidence of over-dosage, and think, as remarked by Cordes (1928), that the onset of such symptoms indicates that toxæmia is already fully established. We have seen occasional cases in which severe toxæmia has developed without any noticeable premonitory sign or symptoms, and Cordes (1928), as well as Roskott and Séno (1928), also record similar cases. In these patients there is probably some cumulative action of the drug for in two of the cases in Series I, of our work, the signs of toxæmia only developed two or three days after the cessation of treatment, and similar cases have been noted by MacPhail (1928), and M'Hutchinson and Duff (1928). If treatment was continued in such cases, severe toxæmia might be expected to occur.

Although cyanosis and epigastric pain are the two most common early symptoms of toxæmia, this condition may in some instances be ushered in by sudden severe vomiting, abdominal cramps, diarrhoea, jaundice, albuminuria, hæmoglobinuria, drowsiness, collapse, etc. Depression, either mental or physical, and weakness are sometimes complained of, loss of weight was observed in some patients and anorexia is not uncommon. Cardiac disturbances of various kinds have been reported, although Muhlens (1927) states he found none in his cyanotic cases. Schulemann and Memmi (1927) reported arrhythmia, but as similar cardiac disturbances have been recorded in malaria *per se*, they were uncertain as to whether these could be attributed to plasmoquine. Tachycardia was noted by Van den Biaden and Henry (1927) and also by Morishita and Namikawa (1928). Bradycardia is considered by M'Hutchinson and Duff (1928) as an additional sign of toxæmia. This condition has also been reported by Baermann and Smits (1929), and Sinton and Bird (1928), and was noted in one of the toxic cases in Series I. It would, therefore, appear that any abnormal disturbance of the heart, greater than can be accounted for by the malarious condition of the patient, should always be considered as an indication for caution in treatment. Severe blood destruction and leucocytosis have been observed in some severe cases of toxæmia, and Muhlens and Weise (1927) in their experiments found that methæmoglobinæmia was more likely to occur in anæmic cases.

The post-mortem findings in fatal cases of plasmoquine poisoning, indicate that the brunt of the toxæmia seems to be borne by the liver and Hulshoff (1928) points out that any indication of liver insufficiency should be considered as a contra-indication to the use of the drug. Muhlens (1927) advises that for the present plasmoquine should not be administered to patients with damaged livers, definite nephritis and cardiac lesions.

#### *Susceptibility to plasmoquine*

Drugs, such as acetanilid and phenacetin, which also contain an alkyl-amino group, have been responsible for a methæmoglobinæmia similar to that in plasmoquine poisoning and idiosyncrasies to these drugs have frequently been noted.

Fischer and Weise (1927) explain the occurrence of cyanosis during plasmoquine treatment as possibly being dependent on some personal idiosyncrasy of the patient. Brahmachari (1928), Namikawa (1928), and several other workers have also taken this view. MacPhail (1928) thinks that the complete absence of symptoms in some patients, while others receiving the same dosage of the drug develop severe toxæmia, suggests such an idiosyncrasy, and Cordes (1928), from his experience of the sudden development of severe toxic manifestations without previous warning, holds the same opinion. Krauss (1929) recommends that 'on account of the very great variation in susceptibility, plasmoquine prescriptions should bear the legend in large type or red ink. "Nil repetantur"'

The question now arises as to the possible explanation of the incidence of such an idiosyncrasy. Menk (1928) states 'it has been the experience in Cuba that Haitian negroes cannot tolerate as large doses of plasmochin as other races'. Hasselmann and Hasselmann-Kahlert (1928) do not believe that the tolerance of the Filipinos to this drug is any greater than in the European. Does a race susceptibility exist? It has struck us as rather peculiar that the number of severe cases of toxæmia seen during our researches, seems to be higher than those recorded in many instances by other workers using the same dosage. The population in our researches was an unusually healthy one, and, except for occasional relapses of chronic malaria, the majority were apparently quite fit in the intervals, so the toxæmia could not be accounted for by any evident physical weakness. Our patients were all of Northern European origin, as were also the severe cases of toxæmia reported by Wade (1929), Ashby (1928) and Squires (1928), and the first author notes specially that he has not observed similar symptoms among Indians receiving the same treatment. When one considers the enormous daily doses, even as high as 0.32 gm., given by some workers to the inhabitants of Southern Europe and of the tropics with few or no recorded severe ill-effects, while severe toxæmia and even death has been recorded in other places after doses as low as 0.06 gm. daily, one is tempted to think that such a racial susceptibility may exist and that possibly it may occur more commonly in persons of Northern European origin among others.

Apart from any factor of individual or racial idiosyncrasy to the drug, the susceptibility of some persons may possibly be explained as due to some temporary organic disturbance of the system, which was not apparent before treatment commenced, such as liver insufficiency, lowering of the alkali reserve, etc. The facts that most of the fatal cases show marked degeneration of the liver post-mortem, and that many of the most severe cases of toxæmia have occurred in patients suffering from malignant tertian malaria, in which disease the effects on the system are more marked than in either quartan or benign tertian fevers, would tend to support such a view. On the other hand Sinton and Bird (1928) were not able to obtain any certain evidence that the administration of glucose and alkali, as prophylactic measures against damage to the liver and a lowering of alkali reserve, caused any decrease in the incidence of toxic symptoms. Brahmachari (1928), however, recommends alkali treatment to ease the abdominal

pains and Manson-Bahr (1928) uses glucose for the treatment of toxic manifestations

One must also think of the possibility of a deterioration of the drug under certain conditions, as a cause of the greater incidence of toxæmia in certain series of patients. Squires (1928) reports that a supply of the drug received by him seemed to give rise to a greater number of toxic cases than the previous supply used. As reported in our record of the toxic symptoms observed in this research, a supply obtained by us about the same time as that received by Squires, seemed to have a similar toxic effect. Although a sample from our stock, when submitted to the makers, was reported to show no increased toxicity, still we think the possibility of increased toxicity should be borne in mind, especially as it is well established that samples of some other synthetic drugs, such as salvarsan, may show an increased toxicity under certain conditions.

The view that many complaints of toxic symptoms are due to auto-suggestion, resulting from the fact that other patients in the same ward or party have complained of such symptoms, seems a possible explanation in some instances, when one considers that mental depression may be one of the features of the toxæmia. The observations that many of the patients who complain of such slight symptoms may later develop cyanosis, if treatment is continued, and that toxæmia may develop several days after the cessation of treatment, is against the view that auto-suggestion is a very common cause of such complaints.

Some observers state that abdominal pains are likely to occur if plasmoquine is given on an empty stomach, or if not followed by a drink of water. Our cases were all given fluid to wash down the tablets and the morning doses were given after breakfast.

It was noted that some of our patients who developed the most severe symptoms were very heavy smokers, but the observations were too few in number to come to any definite conclusion as whether such a habit predisposed to plasmoquine toxæmia.

#### *Recent observations on plasmoquine toxæmia*

In a previous paper (Sinton and Bird, 1928) a summary of the records of toxic manifestations reported by different workers up to that time were given in detail. Since then a large number of other investigators have recorded toxæmia, of varying degrees of intensity in many patients, even although the general average daily dosage of the drug has been greatly reduced.

Muhlen and Fischer (1927) find cyanosis even with daily doses of 0.04 grm. Schulemann and Menz (1927) using a daily dose of 0.06 grm. found that side effects seldom occur, but in 2 cases out of 100 severe cyanosis developed. Morishita and Namikawa (1927) record severe symptoms when doses of 0.06–0.10 grm. were given daily and three out of seven patients with malignant tertian malaria developed hæmoglobinuria. Brosius (1928) with daily doses of 0.06–0.08 grm. found that toxic symptoms occurred not infrequently, but produced no serious permanent effects. On the other hand 15 of his 265 cases developed hæmoglobinuria, and 5 deaths probably due to other causes were reported during treatment. Deeks (1928) says 'it occasionally produces toxic symptoms when given in large doses over a prolonged period'.

Whitaker (1928) after a dosage of 0.06 grm daily reports 6 patients with mild cyanosis out of 40 treated. MacPhail (1928) records 6 toxic cases out of 400 patients treated, when doses of 0.04–0.06 grm daily were given, but says no untoward effects were observed when the smaller dose was used. On the other hand Cordes (1928) reports 6 cases of severe toxæmia out of 250 patients and of these two died. He is of opinion that even small doses of 0.04 grm do not always prevent alarming symptoms. Phelps (1928) found, with a dosage of 0.06 grm that 11 out of 19 patients developed undesirable symptoms and in two instances the reactions were severe. Monk (1928) reports a case of epileptiform fits during treatment with plasmoquine and quinine. M Hutchinson and Duff (1928) with a daily dosage of 0.06 grm had 3 toxic cases among 17 patients. Walravens (1928) records 5 out of 14 cases. De Luca (1928) giving 0.06 grm daily states that tolerance was very good but some symptoms following the use of the drug leave an unfavourable impression. Hulshoff (1928) using the same dosage treated 149 patients of whom three died. In two of these the post-mortem findings could be explained by other causes than plasmoquine poisoning. Drenowsky (1928) reports toxic symptoms apparently of a mild character in 5 per cent of cases. Bhattacharya and Chowdhury (1928) had one patient who developed violent abdominal cramps out of 25 treated with a daily dose of 0.06 grm. Baermann and Smuts (1928) state that there is a risk of intoxication when doses greater than 0.01 grm per 10 kilos of body weight are given as also did Muhlens (1927). Shwensky (1927) with a similar dosage reports 10 per cent of toxic cases. Low (1928) records 3 cases of cyanosis and 1 of abdominal pains. Ignacio (1928) found the untoward effects few and mild. Barber (1928) reports one case of severe cyanosis with a daily dosage of 0.06 grm and De Buen (1928) using the same dosage had to cease treatment in 14 per cent of cases because of unwelcome symptoms. Hasselmann and Hasselmann-Kahlert (1928) observed side effects frequently, but even when giving as large doses as 0.32 grm daily, these were not alarming and passed off quickly when treatment ceased. With daily doses of 0.12 grm they never saw any alarming effects requiring the cessation of treatment. Walravens, Walcke and Bequeret (1928) observed 4 cases of mild toxic effects among 14 patients. Olivier and Hulshoff (1928) observed slight symptoms after a daily dosage of 0.06 grm, while with the same dosage Palma (1928) reports severe cyanosis in 2 to 7 per cent of patients and Wallace (1928) says, although he had many cases with symptoms, none of them were severe. Squires (1928) used a similar dosage for a year without any severe reaction when suddenly 4 cases of severe toxæmia developed, and Namikawa (1928) with apparently the same doses records 9 cases of severe toxæmia among 25 patients. Longo (1928) reports 2 cases out of 30 with slight symptoms. Ashby (1928) had a case with very severe abdominal symptoms, after a total dosage of only 0.16 grm given during 3 days. Krauss (1928) with doses of 0.10 grm and over daily only reports one severe case out of 46 patients, while the same worker (Krauss, 1929) treated 108 patients with a dosage of 0.10 grm with 7 cases of toxæmia, and 27 patients with 0.12 grm with 5 cases. These were mostly slight or transient. Kligler and Reitler (1929) observed one case of chronic malarial hæmoglobinuria and three of definite icterus developing during treatment, and Wade (1929) also reports two severe toxic cases.

In a previous paper (Sinton and Bird, 1928), the records of thirteen serious cases of toxæmia following plasmoquine treatment were collected from the reports of Cordes, Baermann and Smuts, Vad and Mohile, James, Eisberg, Sioli, Fletcher, Manson-Bahr and Sinton and Bird, and amongst these four deaths were included. As will be seen from the records given above, since that time, although the recommended dosage has become very much smaller, yet a large number of cases of toxæmia are still occurring. Cordes (1928) records two deaths certainly due to this drug. Hulshoff (1928) had three deaths during treatment, one of which was probably due to plasmoquine. Brosius (1928)



records that five of his cases died during treatment. The post-mortem appearances found in most of these suggest that death was due to causes other than plasmoquine poisoning, yet it seems possible that the fatal issue may have to some extent been accelerated by the drug.

It is also of interest that although plasmoquine as compared with quinine has been vaunted as the treatment of choice in blackwater fever, yet several workers have recorded the occurrence of hæmoglobinuria commencing during treatment with this drug.

#### *Toxic symptoms observed in the present research*

When one takes into consideration the fact that in many of the reports on plasmoquine, numbers of the patients on whom treatment with this drug had been tried, were of poor physique, badly nourished and sometimes cachectic, it is not surprising that cases of toxæmia are recorded, although some observers think that the drug is as well borne by such subjects as by stronger persons. Mühlens (1927), however, states that great care should be used in the dosage of weakly individuals, and Fischer and Weise (1927) found that methæmoglobinæmia was greater in anæmic patients. The population used in our work, however, were young well-nourished British soldiers, living under very good conditions who, except when an acute attack of malaria was occurring, appeared to be in the very best of physical condition. With such a comparatively healthy population, of an average weight of about 10 stone (63 kilogrammes) each, one did not expect to get many toxic symptoms with doses of 0.06 gm. or less of plasmoquine daily, yet these were found to occur.

In Series I which received 0.06 gm. of plasmoquine daily, although toxic symptoms such as slight epigastric pain or cyanosis were observed while the original consignment of plasmoquine was in use, yet on no occasion did this give rise to any anxiety and the symptoms quickly disappeared with a few days' rest. When this lot of plasmoquine was finished a new consignment received in March 1928 was taken into use on 28th June, 1928, and within a few days afterwards six out of the thirteen patients being treated developed toxic symptoms of a greater or lesser severity. One patient received 3 days' treatment without symptoms, ~~another received 4~~ days of treatment and of these three developed toxic the treatment and two others after treatment had been patients who received 5 days' treatment one developed two patients who had 6 and 7 days' treatment showed and that out of 13 patients who had received a total of average 5 days each, with the new plasmoquine that 6 cases severe, inside 5 days after commencement of the patients who had received a total of 167 days of treatment (average 13 days per case), only 5 developed toxæmia that only of a mild character.

The symptoms, etc., in the three severe cases are summarized below —

*Case B G*—No symptoms reported until the 5th day of treatment (all with new plasmoquine), when he complained of severe and constant epigastric pain with marked anorexia. He vomited twice after admission to hospital. Considerable cyanosis. Bradycardia (pulse 44). Plasmoquine treatment stopped immediately. On the 6th day pain less severe but cyanosis more marked. On 7th day pain again more severe. Urine showed no albumen. On 8th day, pain much less, but cyanosis persists, appetite returning. The cyanosis remained until 10th day, i.e. for six days after plasmoquine was stopped.

*Case G H*—No toxic symptoms reported until 15th day of treatment (5th day of new plasmoquine), when he complained of very severe cramping epigastric pain. Vomited once. Slight cyanosis. Treatment stopped. On the next day the pain was less but cyanosis persisted for several days.

*Case S H*—During the last 4 days of his course of treatment, he received the new plasmoquine. After an interval of one day from the cessation of treatment he complained of severe epigastric pain and showed slight cyanosis. On the next day the pain was more marked and the cyanosis continued. On the 4th day after treatment stopped the pain was less severe and the symptoms had completely disappeared next day.

*Case G H*—Received the new plasmoquine for the last 4 days of his course of treatment. On the 4th day after treatment stopped he was again admitted to hospital with slight cyanosis and complained of complete anorexia. He was found to have lost 3 pounds in weight during the previous week. His symptoms disappeared after two days in hospital.

The sudden outbreak of more toxic symptoms following the use of a new sample of the drug gave rise to some anxiety and treatment was discontinued. Although the occurrence might be a coincidence, yet it was thought possible that either there might be some deterioration of the drug from storage in the tropics or else that there might be differences in toxicity in different samples of the drug manufactured. The possibility of the latter factor was especially considered because Squires (1928) reported that certain samples received by him at Khar-toum early in 1928, seemed to be more toxic than previous samples. Samples of the drug were returned to the makers for testing, but they reported that they were unable to find any increase in the toxicity of the samples sent, so we are unable to account for the apparent greater toxicity of this sample as compared with previous ones.

Of a total of 17 patients treated with 0.06 gm plasmoquine daily in combination with quinine, eleven or 64 per cent showed toxæmia of varying degrees of intensity, so it would seem that for continuous treatment a dosage of 0.06 gm daily is too high for routine practice. It is curious that other workers using this or larger doses do not record so many toxic cases nor such a high percentage with marked toxæmia. It is possible, as discussed above, that this may be due to some racial idiosyncrasy among the patients treated by us, as there was no apparent physical disability among the patients to account for it.

In Series II where the daily dosage was 0.04 gm, only 12 or 25 per cent of the 48 patients treated showed any toxic symptoms. In eleven instances these were slight abdominal pain and in one case slight cyanosis. This dosage does not seem to produce marked signs of toxæmia in robust adults.

In Series III where 0.03 gm was given daily by intramuscular injection in no case was toxæmia observed.

## THE USE OF PLASMOQUINE OUTSIDE HOSPITAL PRACTICE

There are apparently two diametrically opposite schools of opinion on the question as to whether plasmoquine should be used under conditions which are not under strict medical control

The opinions quoted below express the view that the drug can be used for the treatment of patients, who are not under constant medical supervision or even that it can be given to the lay public for administration —

Nutter (1927) states that he 'can see no objection to issuing plasmochin compound for treatment outside hospital' Schulemann and Memmi (1927) think that ambulant cases are less liable to develop cyanosis than hospital patients on the same dosage Benecke (1927) believes that the administration of this drug can unhesitatingly be handed over to laymen Deeks (1928) states 'from two years' experience, we now know its limitations fairly well, and feel confident that its use can be extended to the treatment of camp cases in such dosage as to be effective without causing serious results' MacPhail (1928) found that 0.04 grm plasmoquine for 6 days was not followed by untoward symptoms and therefore thinks 'our experience justifies the conclusion that plasmoquine in this dosage in combination with quinine can safely be administered under very ordinary supervision' Barber (1928) states that plasmoquine compound seems safe for field work Wallace (1928) says that 'it can be used with safety in the field in the doses mentioned,' i.e., daily doses of 0.04 grm plasmoquine and 0.5 grm quinine Krauss (1928) who was using the drug in doses of 0.10 and 0.12 grm daily says that plasmoquine compound seems safe for field work and for administration in public health clinics Barber (1929) reports that in the plantations of the Panama Division of the United Fruit Co., the negroes are given a two-days' supply of plasmoquine and quinine to take home and asked to return at the end of two days for a further supply They are, however, warned that if toxic symptoms develop they should stop treatment immediately and obtain medical advice The makers in one of their pamphlets apparently sanction the use of plasmoquine treatment outside strict medical control, for they state that 'the continuous treatment is principally indicated in out-door patients especially among the working classes, when interval treatment, which has been found very successful, meets with difficulties'

The opinions recorded above would seem in our opinion to minimize the dangers of a drug, the use of which we believe to be still in the experimental stage Other workers, however, do not agree with the views expressed above —

Sinton and Bird (1928) state that 'if such (toxic) events have occurred under the strict and careful conditions in which the drug has been tested, it seems to us that the time has not yet arrived when it can be given broadcast for use in general practice, however useful it may be under hospital conditions or under very strict and *daily* medical supervision In the present state of our knowledge of the action of this drug it is essential, in our opinion, that every patient under this treatment should be seen at least once daily, so that administration can be stopped as soon as the first signs of toxæmia are detected' 'Before the drug can be taken into general use, much more work is necessary under carefully controlled conditions to determine the best dosage and the duration of administration' Brosius (1928) also emphasizes the fact that the use of the drug is still in the experimental stage Olivier and Hulshoff (1927) think it should only be given under strict medical supervision Cordes (1928) states that, for therapeutic as well as for prophylactic purposes, the toxic effect of plasmoquine constitutes a lamentable drawback, and goes on to say that 'it does not seem safe to place plasmoquine in the hands of dispensers or rural overseers for free distribution' 'Its administration requires a constant control of the patients which can be carried out effectively only in hospital Plasmoquine should not be given without permanent medical control, even small doses of 4 cgrms do not always prevent alarming symptoms' Phelps (1928) gives

as his opinion that 'plasmoquine is a dangerous drug and, as the reaction and susceptibility of an individual cannot be predetermined by any means at present familiar to us its use will usually be restricted to hospital cases. Roskott and Seno (1928) advise that the drug should remain in medical hands and treatment carried out in clinics. Hasselmann and Hasselmann-Kahlert (1928) state that 'these possible *side effects make medical supervision absolutely indispensable*' and plasmoquine unfit for self-treatment after treatment or prophylaxis without medical care. Nankawa (1928) thinks that the drug should not be trusted to the lay. Bachmann and Smits (1929) say that 'in its present form it is unsuited for mass treatment in view of its grave or deadly effects in advocated doses its administration demands the utmost vigilance. Krauss (1929) advises that 'on account of the very great variation of susceptibility, plasmoquine prescriptions should bear the legend' '*Nil repetitur*' in large type or red ink and the patients should remain under daily observation. If this cannot be done the remedy should not be prescribed'. Kligler (1929) says that 'for the time being one ought to limit the use of plasmoquine to treatment under the control of a physician and not use it wholesale as a prophylactic. Kligler and Reitler (1929) record the results of their prophylactic use of the drug in Palestine and state that 'these facts indicate that great caution should be observed in administering the drug *en masse* and that until the methods and dosage are carefully worked out the work should be carried out only under the constant supervision and control of a physician. The results indicated that extreme caution must be observed in mass treatment with this drug'.

The results of our work with daily doses of 0.06 gm of the drug indicate that, even in a robust adult population under strict medical control, toxic symptoms of greater or less severity are not uncommon, but that with daily doses of 0.03 or 0.04 gm of the drug, these symptoms are rarer and less marked, but Cordes (1928) reports severe toxæmia even with a daily dose of 0.04 gm. If such a population living under comparatively ideal condition of housing, feeding and absence of hard work, are liable to exhibit toxic symptoms, it appears to us that such symptoms may be expected to be commoner and more severe amongst populations under less favourable conditions such as are found widespread in malarious countries. It may possibly be, as suggested above, that our patients being Northern Europeans are more liable to develop toxic manifestations than persons of more tropical climates. Until we have some more definite indication as to what may be considered a dosage which can be administered without toxic symptoms, irrespective of race, and which will at the same time give a curative effect, it does not seem to us advisable that the drug should be issued for general use to the lay public, but that it should only be given under medical supervision. It may be that smaller non-toxic doses will require a longer period of treatment to effect a radical cure, but this would not be of such importance if a dose could be determined which would be safe for general use, for one must remember that a large proportion of the population in many malarious areas of the world are not within the reach of medical aid. The occurrence of many cases of toxæmia among the lay public would tend to bring into disrepute what is undoubtedly a very valuable drug in the treatment of certain forms of

## CONCLUSIONS

The time has not yet arrived when plasmoquine can be issued for use except under the *constant supervision and control of the medical profession*. It, however, seems possible that a safe dosage and method of treatment for more general use may shortly be determined.

The conclusions we arrived at as the result of our work were —

(a) The combination of quinine with plasmoquine is better than either drug separately, in the production of a permanent cure in chronic benign tertian malaria.

(b) Plasmoquine is a very important adjuvant to quinine treatment and should not be used except in combination with this drug.

(c) The daily dosage of quinine to be given with plasmoquine should not be less than 1.25 gm (20 grains).

(d) Continuous treatment with plasmoquine, in small doses combined with quinine, produces a greater number of permanent cures than larger doses given by the interrupted method and is less liable to produce toxic symptoms.

(e) Doses of plasmoquine greater than 0.04 gm daily should not be given, and possibly even 0.03 gm or less will be found to be the maximum safe daily dose. This remains to be determined.

(f) A more prolonged course of treatment with small doses of plasmoquine would seem to be a better treatment than larger doses given over a shorter period.

(g) Plasmoquine treatment should be stopped on the least suspicion of the occurrence of toxic manifestations.

(h) Plasmoquine treatment should not be given, or only with extreme caution in hospital, to persons suffering from lesions of the liver, kidneys and circulatory system. Great care should also be exercised in the treatment of anæmic and weakly individuals.

Our thanks are due to the Director of Medical Services in India and the Officer Commanding the British Military Hospital, Kasauli, for the facilities which they have placed at our disposal for carrying out this investigation. We also wish to thank the Indian Research Fund Association for the funds which they provided to cover the expenses entailed.

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# STUDIES IN MALARIA, WITH SPECIAL REFERENCE TO TREATMENT

## Part XIII.

### PAROSAN AND DIMEPLASMINE IN TREATMENT

BY

MAJOR J A SINTON, I M S  
(*Malaria Survey of India, Kasauli*)

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SINCE the therapeutic effects of stovarsol and plasmoquine in malaria were discovered, the makers of these drugs have been attempting to improve upon them. Messrs May and Baker, London, have asked us to test the effects of parosan oxide, a new arsenical preparation, and its compound, quinine-parosan, in the treatment of malaria, while the Haverø Trading Company, Calcutta, the agents for the I G Farbenindustrie Aktiengesellschaft who make plasmoquine, have supplied us with a small quantity of dimeplasmine.

Unfortunately it has only been possible to try these drugs on a few patients, as the failure of the monsoon in a large part of Northern India during 1928 with the consequent marked diminution in malarial incidence, has resulted in a great decrease in the numbers of malarial patients available for treatment.

#### PAROSAN OXIDE

The composition of this drug as given by the makers is 8-acetylamino 3-hydroxyl 1-4-benzisoazine 6-arsenous oxide, the parent substance, parosan, being the corresponding arsenic acid.

The makers report experiments which show that it is practically non-toxic to mice, while against infection with *Trypanosoma equiperdum* in this animal its curative value is marked.



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The agents state that the drug has been tested at Hamburg, where it was found that eight daily doses of 0.25 gm., i.e., 2.0 grms of the reacidified salt or 0.8 gm of the base, while having no effect on malarial parasites, was at the same time non-toxic.

The drug has been issued to us in tablets each weighing 0.4 gm and containing 0.25 gm of the reacidified salt of the base, each equal to 0.1 gm of the base.

#### TRIAMINIS USED AND RESULTS RECORDED

A similar procedure, as that described in previous articles of this series, was employed to ensure proper administration of treatment and detect relapses. All the patients showed parasites in their peripheral blood before treatment was commenced.

#### PAROSAN OXIDE AND QUININE-PAROSAN

Parosan oxide was given in doses of 6 to 9 tablets (0.6—0.9 gm) daily for 14 days. As in previous work with such arsenical compounds, an alkaline mixture and sugar diet was given daily to protect the liver from possible arsenical poisoning. Under these circumstances, no toxic symptoms were observed.

One patient with malignant tertian malaria and five with chronic benign tertian were treated in this manner. Of these, the case of malignant tertian malaria relapsed on the 10th day of treatment. Three of the benign tertian patients completed 8 weeks of blood observation after treatment without relapse and one patient relapsed on the day after treatment stopped. The remaining patient showed parasites continuously for 9 days, after which time parosan treatment was discontinued and plasmoquine started, followed by a quick disappearance of parasites from the peripheral blood. The failure rate was therefore 40 per cent, but the numbers observed are small.

Four patients suffering from chronic benign tertian malaria were given quinine-parosan in doses of 6 tablets daily for 14 days, corresponding to 0.6 gm parosan oxide and 0.9 gm quinine sulphate daily. The same precautions as those mentioned above were taken against arsenical poisoning. This dosage seemed to be too small, for all the patients relapsed, one during treatment, two immediately after it was stopped and the last at the end of 6 weeks of observation.

In view of the above results, a second series of ten patients with the same type of fever were given 9 tablets (0.9 gm parosan oxide, 1.35 gm quinine sulphate) daily for 14 days. Three of these patients relapsed and the remainder completed observation without relapse.

The relapse rate in the latter series was therefore 30 per cent, while with a similar dosage of quinine troposan the rate was about 40 per cent (Sinton, Bird and Orr, 1928).

*Conclusions*—(a) The number observed is too small from which to draw any definite conclusions as to the relative efficiency of parosan and troposan in the production of a permanent cure in malaria. (b) The results suggest that parosan may be as effective as troposan when used in combination with quinine and that,

because of the absence of toxic symptoms, larger doses might be tried with safety

### DIMEPLASMINE

Six patients suffering from chronic benign tertian malaria were treated with this drug. One patient received 6 pills daily for 7 days but he was lost sight of at the end of treatment, so nothing is known about the permanent effects of the treatment.

Four patients received 9 pills daily for 19 to 21 days and of these two relapsed and two completed eight weeks of observation without relapse. The remaining patient received 9 pills daily for 4 days when the supply of the drug available was exhausted, and his treatment was continued with plasmoquine, after which he did not relapse. No toxic symptoms were observed with the dosage of the drug used.

*Conclusions*—It has not been possible to arrive at any conclusions from the results obtained.

### DURATION OF PARASITES IN THE PERIPHERAL BLOOD

The bloods of all the patients under treatment with these drugs were examined daily by the thick-film method and the results are recorded in Table I, in which is given also the results of previous work with quinine-troposan, plasmoquine and plasmoquine compound for comparison.\*

As far as can be judged from the few cases observed it would seem that neither parosan oxide, quinine-parosan nor dimeplasmine are as effective as either quinine-troposan or plasmoquine compound in clearing the peripheral blood of *P. vivax*, but that their value is almost the same as that of plasmoquine alone.

One patient having a mixed infection of *P. vivax* and *P. falciparum* was also treated with 9 pills of dimeplasmine daily. With this drug the former parasite disappeared inside 24 hours, while the latter was still present at the end of 7 days when it was found necessary to start quinine treatment.

### DURATION OF FEVER

The same procedure for the recording and estimation of fever was employed, as in previous work.

The average duration of fever found with parosan oxide treatment was 0.66 days (maximum  $2\frac{1}{2}$  days), with quinine-parosan 0.82 days (maximum 3 days).

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\* As the cases treated were all chronic infections, it should be pointed out that the tests of both 'duration of parasites' and 'duration of fever' have not, in my opinion, the same value as when these conditions are observed in fresh infections, for it is well known that both parasites and fever will disappear more or less rapidly in most chronic infections if the patient is merely given rest in bed, although no specific parasiticidal treatment is given.

TABLE I

Treatment	Average duration per case days	Number of patients observed	NUMBER AND PERCENTAGE OF PATIENTS SHOWING <i>P</i> 2407 AFTER HOURS --				
			0	24	48	72	96
QPO	15	14	14 (100 per cent)	8 (57.1 per cent)	6 (43 per cent)	4 (28.5 per cent)	3 (20.7 per cent)
PO	12	6	6 (100 , )	5 (83.3 )	2 (33.3 " )	1 (16.6 , )	0
DMP	14	7	7 (100 , )	4 (57.1 " )	2 (28.5 " )	2 (28.5 )	1 (14.5 )
QT	0.50	38	38 (100 , )	15 (39.5 " )	3 (7.9 )	1 (2.6 )	0
PM	17	46	46 (100 , )	42 (91 " )	26 (56 , )	8 (17 , )	3 (6.5 )
PMc	0.71	34	34 (100 )	18 (53 " )	6 (17 " )	1 (3 , )	0

QPO = Quinine-troposin, PO = Plasmocin, DMP = Dimepharmine, QT = Quinine-troposin, PM = Plasmocin, PMc = Plasmocin

and with dimeplasmine 0.58 days (maximum  $1\frac{1}{2}$  days) When these are compared with the previous records of 0.34 days with quinine-troposan, 0.37 with quinine-stovarsol, 0.55 with plasmoquine compound and 0.30 with the cinchona alkaloids, it would seem that neither parosan oxide, quinine-parosan nor dimeplasmine are as effective in the reduction of fever as the other drugs, combined with quinine, which have been tested previously The results may however be influenced by the small numbers of patients treated

My thanks are due to Major S. Smith, R.A.M.C., and Captain W. B. F. Orr, R.A.M.C., for their assistance in carrying out these tests, and to Messrs May and Baker, London, and the Haverro Trading Co., Calcutta, for the drugs used

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# THE EPIDEMIOLOGY AND PATHOLOGY OF TUBERCULOSIS IN INDIA

BY

A C UKIL,

*Professor of Bacteriology, National Medical Institute and Officer-in-charge,  
Tuberculosis Research Indian Research Fund Association, Calcutta*

## INTRODUCTION

THE earliest attempt to map out the prevalence of tuberculosis in India was made by Lankester(1) nearly a decade ago, but his observations were chiefly based on statistics furnished by official and non official sources. Liston and Soparkar(2) tried to find out the types of tubercle bacilli responsible for surgical tuberculosis in India and the susceptibility of Indian milch cattle to tuberculosis.

In the present inquiry an attempt has been made to find out the degree of tubercular infection or tuberculization among the people by cuti-reaction according to environment, age, sex and occupation in urban and rural areas, from radiographic observations, post-mortem evidence and hospital cases. The frequency and incidence of the various types of the disease have also been tried to be determined from clinical evidence on cases, post-mortem evidence on hospital cases and cases of accidental death autopsied at the Police Morgue, histological and bacteriological study of the various lesions in tuberculosis and the typing of tubercle bacilli isolated from the pus of surgical tuberculous lesions. The pathology of the disease has been considered only so far as it concerns our epidemiological studies.

To understand the epidemiology of tuberculosis in India, we ought to remember that 94 per cent of India's population reside in the villages, that urbanization and introduction of rapid transport facilities in India have been a recent development and that there are still large tracts of country not penetrated by railways or other rapid methods of communication. The epidemiology must, therefore, vary in different parts of the country. We have, therefore, carried our investigations in urban, rural as well as in well-isolated areas away from rail routes in Bengal, Behar, Assam and Madras, so that the different areas represented varying degrees of urbanization.

Tuberculosis has been a prevalent disease in most big towns in India from ancient times but there is evidence that the disease has been rapidly spreading

and quickly proving fatal in recent years in all parts of India, e.g., there has been an increase of 77 per cent in mortality from tuberculosis in Calcutta within the last 10 years. In addition, there are certain social factors which are operating in determining the epidemiology of the disease, the chief of them being the following —

(1) Habits—the habit of spitting inside habitations, eating and drinking from common vessels and sleeping together in the same room are extremely common in all strata of society and are powerful factors in offering facilities for massive infection.

(2) Customs—Early marriage, early motherhood and the purdah system account for the increased incidence and death-rate among females. The death-rate among females between 15—20 years is 6 times that of males of the same age within the municipal limits of Calcutta.

(3) Diet—The bulk of the people take a badly balanced dietary poor in vital ingredients.

#### EPIDEMIOLOGY

##### *The von Pirquet reaction as a means of mapping out tuberculous infection in the community*

It is difficult to estimate the number of people infected with or suffering from tuberculosis from hospital returns or vital statistics alone. A more definite method has come to be in use since von Pirquet described the cuti-reaction in 1902. Very interesting and useful studies have been made in various countries which have helped us not only to know the degree of infection among the people of a country but also to understand the character of pathological changes met with in various lesions.

##### *Technique and interpretation of the test*

In doing the test pure Tuberculine Brute prepared at the Pasteur Institute, Paris, has been mostly used for obtaining comparative data in other parts of the world. The skin of the forearm is cleansed with absolute alcohol, allowed to dry and with separate platinum loops a drop of 0.5 per cent carbolyzed saline (as control) and a drop of tuberculine are consecutively placed on the skin at a distance of 2 inches. Equal number of superficial scratches are then made with a vaccinating lancet and the areas are then allowed to dry.

Reactions were recorded, after an interval of 48 hours, as positive if there were redness and palpable (between 2 fingers) oedema in and around the scarified area. The intensity of the reaction was noted under 4 heads—strong (indicated by +++ ) when the diameter equalled or exceeded 1 cm, moderate (indicated by ++ ) when the diameter was between 0.5 cm—1.0 cm, weak (indicated by + ) when the diameter was below 0.5 cm and doubtful (indicated by ± ) in estimating the number of total positives, half the number of doubtful cases was included.

*Summary of cuti-reaction data*

In all, 6,500 persons have been tested of which 4,279 individuals were tested in Bengal, 1,064 in Madras, 617 in Sylhet (Assam) and 540 in Ranchi and Hazaribagh (Behar). The data first obtained in Bengal essentially agreed with those obtained in other provinces and it is believed the same thing holds good in other parts of India.

The infants and children were mostly obtained from the child welfare clinics and children's wards of the hospitals in Calcutta, children between 6—15 years were chiefly obtained from the schools in cities and rural areas. Individuals from 16 years onwards were obtained from college students, clerks, prisoners and mill hands. Thus an opportunity was obtained to study the influence of environment in the diffusion of tuberculous infection. The following tables will illustrate the various aspects of the question. The details of provincial distribution are given in the Appendix.

*Cuti-reaction according to age and intensity of reaction*

Age	INTENSITY OF REACTION					Total number tested	Per cent positive
	+++	++	+	+	—		
0—5 years	1	17	19	3	296	336	11.4
6—10	1	38	178	57	539	813	30.1
11—15		36	99	58	314	507	33.3
16—20	10	70	166	83	425	754	38.1
21—25	23	91	279	114	393	900	50.0
26—30	43	125	308	166	418	1,060	52.7
31—40	68	132	454	156	497	1,307	56.0
41—50	24	56	164	98	152	494	59.4
51—60	14	17	100	34	62	227	65.2
Above 60	12	15	35	18	22	102	69.6
TOTAL	196	597	1,802	787	3,118	6,500	45.9
Per cent	3	9.1	27.7	12.1	47.9		

Considering that all the infants and children of the age group 0—5 years belonged to a highly endemic area like Calcutta, the percentage of positive reactions is strikingly low. Fifteen out of 38 of them were found to have been in contact with 'open' pulmonary tuberculosis cases in the family. We did not get a positive reaction in infants below 6 months. The highest sensitization seems



to occur at the 30th year, when the incidence of all forms of tuberculosis is the highest also. The average bacillization of 45.9 per cent of a mixed population in India is in striking contrast to data in Europe. A comparative study of figures in other Asiatic countries is instructive.

*Comparative data in other Asiatic countries*

Age	Bengal Per cent	Cochin China (Lalung Bonnaire) Per cent	Indo China (De Langen) Per cent	Java (De Langen) Per cent	France (Marfan) Per cent
At 10 years	30.1	35—45			63.7
„ 15 „	33.3	64—75			81.9
„ 20 „	38.1	76—89			89.0
Average	45.9	67.0	65.0	65.0	

We do not know whether Cochin China, Indo-China and Java differ from India in matters of urbanization or whether only urban people were tested. A high figure in urban areas in India has been brought down to an average medium figure by the low incidence of tubercularization in people of rural areas.

The low percentage of positive reaction in infants and children disappears in presence of massive infection in the family. Out of nearly 100 tubercular homes surveyed, through the help of the Baby Welfare Clinics and of the newly started Bengal Tuberculosis Association in Calcutta, we found the incidence of cuti-reaction as follows —

*I Living in the same room with a tubercular patient*

Age between 1—5 years = 71.4 per cent positive  
 „ „ 6—10 „ = 83.3 „ „

*II Living in the same house but not in the same room*

Age between 1—5 years = 50 per cent positive  
 „ „ 6—10 „ = 58 „ „

The question of a low degree of bacillization in presence of a low mortality figure requires further investigation and is engaging our attention.

*The significance of the intensity of a positive reaction*

The intensity of a reaction indicates the strength of the allergic state or immunological response of the cells of the body to tuberculo-protein—the stronger the reaction the better the response. The reaction is due to previous infection with bacilli and it must not be taken as evidence of tuberculous disease.

We have found that in the ordinary course of urbanization a person begins to give a positive reaction in 2 to 3 years after residence in a thickly populated city or town. With the rural people, when they give a positive reaction, it is also a

weak one. We have come to look upon weak reactions as imperfect immunological response on the part of the body. Strong reactions are most frequent between the 20th—40th year when all forms of tuberculous disease are most common also. If a rural individual or a child under 10 years gives a strong (+++) reaction, we look on it as an evidence of exposure to massive infection usually in the family, for we have traced many 'open' pulmonary cases in this way. Such evidence can also be obtained from the moderately strong (++) reactions but to a less extent. Infants and children living in tuberculous homes in close proximity to the patient usually show fairly well-marked reactions.

In spite of harbouring living tubercle bacilli or antigen in the body, a cuti-reaction may be negative due to 4 causes —

- (1) The dose of tuberculin has been too small to wake up a reaction,
- (2) the reaction has been done during the ante-allergic (or incubation) period,
- (3) the individual is non-immunized, and
- (4) where in spite of active disease, the immunity is depressed and the immunological response fails to appear in such conditions as advancing tuberculosis with relatively massive re-inoculation, pregnancy, extremely low states of general health and acute infections like influenza, measles, whooping cough and cachexias.

The figures given below show where the intensity of reaction helped us to spot out 'open' pulmonary tuberculosis cases in the family —

Age	Intensity of reaction, per cent positive					REMARKS
0—5 years	100	85			1	Six cases who were to have lived in the same house gave a negative reaction
6—10 ,	100	24	12		5	
11—13 „	50	8	5			

Thus a strongly positive reaction in infants and children up to 10 years is an indication of exposure to massive infection, usually in the family. A moderately positive reaction is so indicative chiefly in young children between 0—5 years. A strongly positive reaction in an individual should always give rise to suspicion of a source of massive infection. In presence of an apparent tubercular lesion, such a reaction is, therefore, strongly suggestive of a positive diagnosis.

*The influence of habitation on cuti-reaction in urban and rural areas*

The influence of habitation on tuberculization in urban and rural areas has been shown separately for Bengal and for the four provinces combined in the following figures and curves which are self-explanatory.

Analysis of cuti-reaction according to class of people tested

Age	INFANTS AND CHILDREN, 336 TESTED		MALE STUDENTS, 1,533 TESTED		FEMALES, 851 TESTED		PRISONERS, 3,531 TESTED		MILL HANDS, 250 TESTED	
	Per cent positive	Number tested	Per cent positive	Number tested	Per cent positive	Number tested	Per cent positive	Number tested	Per cent positive	Number tested
0—5 years	11.4	336								
6—10 "		.	29.5	572	31.7	241				
11—15 "			29.7	321	36.5	186				
16—20 "			29.9	384	32.3	88	49.3	231	58.0	51
21—25 "	..		37.6	174	36.2	40	52.8	625	65.5	61
26—30 "			41.4	82	48.0	52	52.6	874	76.0	52
31—40 "	..				68.3	131	53.6	1,124	74.5	52
41—50 "	..				71.4	63	56.0	405	82.0	26
51—60 "	.	.			72.7	11	64.1	208	81.0	8
Above 60	..			.	73.6	38	67.1	64		

CHART 1

THE INFLUENCE OF HABITATION ON TUBERCULIZATION IN INDIA  
(ON 6500 PERSONS IN BENGAL, MADRAS, BEHAR & ASSAM)

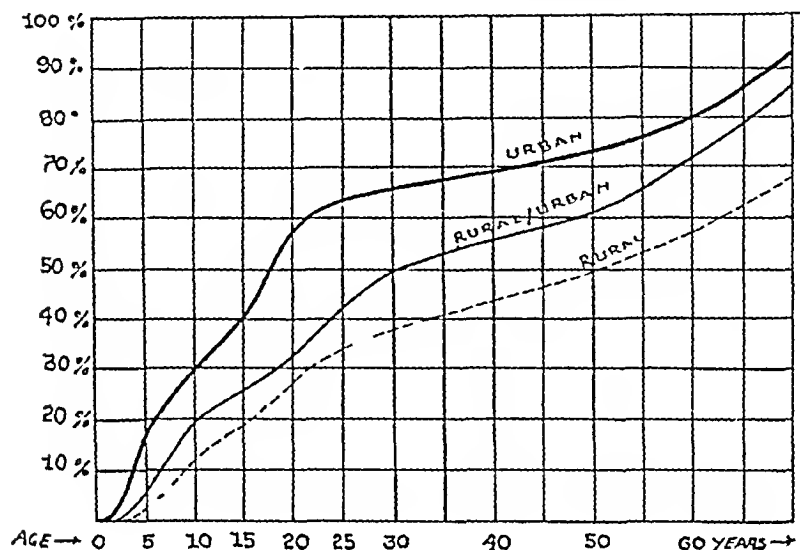
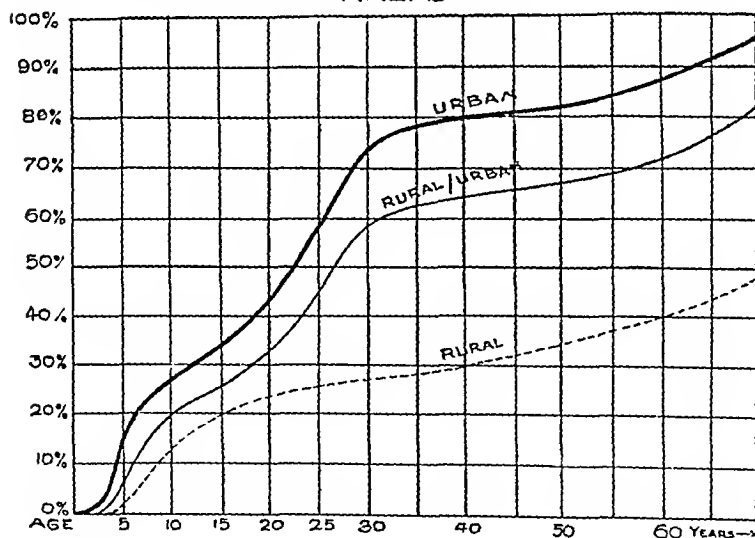


CHART 2

THE INFLUENCE OF HABITATION ON TUBERCULIZATION  
IN BENGAL  
ON 4300 PERSONS IN RURAL, URBAN AND INDUSTRIAL  
AREAS



The above curves may be depicted in figures as follows —

*The influence of habitation on cuti-reaction*

Age	Rural Per cent	Rural/Urban Per cent	Urban Per cent
0—5 years			11·4
6—10 „	15 0	20 0	30·0
11—15 „	19·6	26 0	41 0
16—20 „	28 8	33·0	58·3
21—25 „	34 0	43 0	64 0
26—30 „	38 0	50·0	66 0
31—40 „	43·7	56 0	69·0
41—50 „	50 0	62 5	73·0
51—60 „	57 3	72·0	80·0
Above 60 „	66 6	Number too small	Number too small
Average (per cent)	33 4	47 9	55 1

*The reaction according to habitation in different classes of people*

According to habitation	Students (6 to 30 years)	PRISONERS (16 TO 70 YEARS)				Mill Hands (16 to 60 years)
		Bengal	Madras	Ranchi and Hazaribagh (Behar)	Sylhet (Assam)	
Rural (per cent)	21 8	32·1	33 7	21 0	69·0	69·0
Rural/Urban (per cent)	30·1	50·4	46 1	22·2	78 9	70·0
Urban (per cent)	32·3	74 1	56 5	23 8	77 8	76·0

It has been found that rural people with negative cuti-reaction begin to give positive reactions after a stay of 2 to 3 years in highly endemic areas like Calcutta. Such positive reactions are always weak. Rural people, in most cases, give a weak reaction.

A long residence in a Central Jail reacts like residence in a big town. That is, the chances of diffusion are greatest where there is a large aggregation of people.

*Cuti-reaction according to occupation*

Most of the cases quoted below were prisoners in jail. The incidence will be noticed to be particularly high in carters, tailors and mill hands.

*The influence of occupation on cuti-reaction (Jail cases)*

Occupation	Total number tested	Total number positive	Percentage positive
Cultivator	2,456	1,017	41.4
Mill hands	176	121	68.7
Carters	89	65.5	73.5
Tailor	22	15.5	70.4
Servant	296	192	64.8
Coolie	87	44	50.5
Carpenter, mechanic, goldsmith etc	128	80.5	62.8
Shop keeper, businessman	456	283.5	62.1
Clerk, school master	131	74.5	56.8
Jail life	197	134.5	68.2
Beggar, vagabond	43	24	55.8
Landlord	10	6.5	65.0

*Cuti-reaction according to religion*

Religion	Total number tested	Total number of positive reactions	Per cent positive
Hindus	4,190	1,810	43.2
Mahommedans	2,194	1,135	51.7
Christians	116	43.5	37.5
TOTAL	6,500	2,988.5	45.9

The Mahommedans show a slightly higher incidence, but this is due chiefly to the higher incidence in urban cases. The apparently low incidence in Indian Christians may be due to the small number tested. No race susceptibility can be proved from these figures.

*The relation between physical build and cuti-reactions*

Individuals were arbitrarily divided into three classes according to their physical build and musculature. It will be seen from the figures that those with poor physique show a more extensive sensitization to tubercular infection.

State of health	Total number tested	Total number positive	Per cent positive
Good	2,757	1,227	44.5
Indifferent	2,978	1,358.5	45.6
Bad	765	403	52.6
TOTAL	6,500	2,988.5	45.9

*The relation between gland incidence and cuti-reaction*

One thousand seven hundred and sixty-four individuals (chiefly infants and children) were examined for the presence of palpable neck glands. 53.2 per cent were found to possess palpable neck glands.

Age	Number showing palpable glands tested	Number showing positive cuti-reaction	Per cent positive
0—5 years	151	31	20.5
6—10 „	541	160.5	29.6
11—15 „	135	41	30.3
16—20 „	94	30	31.9
21—25 „	18	4	22.2
26—30 „	1		
TOTAL	940	266.5	28.2

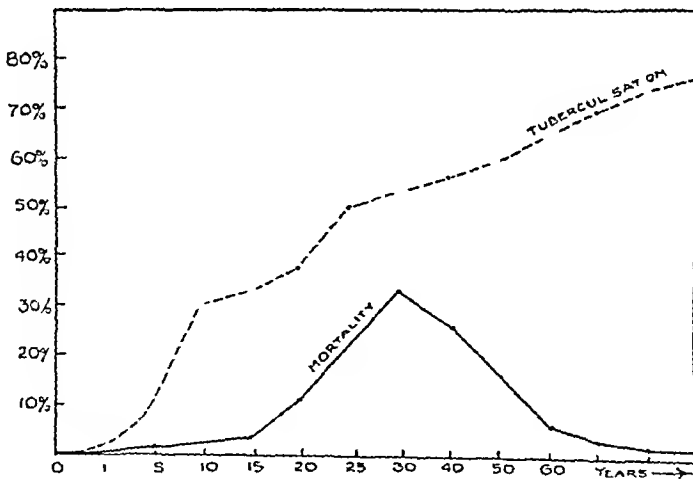
The glandular enlargement in the majority of cases must therefore be accounted for by other conditions in the mouth, naso-pharynx and scalp. Some workers, e.g. Frankster(1), were tempted to call all enlargement of cervical glands as due to tubercular infection. Cases with enlarged glands of the posterior auricular, occipital, submental and submaxillary groups were not counted if there was a manifest septic focus to account for them.

*The relation between tuberculous mortality and diffusion of tuberculosis*

The mortality curve depicted below has been drawn from the figures of 5 years' death rates in Calcutta, as it is useless to look for reliable data outside the principal municipal areas. A feature in this curve is the low mortality figure in infancy, in the presence of a low degree of bacillization as shown by cutireraction. Two curves are reproduced from a paper by Sir Arthur Newsholme(5) showing the mortality rates in England for the sake of comparison. The Registrar General's annual report for 1926 in England and Wales showed that tuberculosis was responsible for 8.3 per cent of the total deaths and that 14.5

CHART 3

RELATION OF TUBERCULOSIS MORTALITY (CALCUTTA)  
TO DIFFUSION OF TUBERCULOSIS  
IN EASTERN INDIA



per cent of the total number of deaths from tuberculosis occurred in children under 15 years. The susceptibility to tuberculous disease is uncommon during the first eight months of life but it rapidly increases for a year and a half after this when it begins to decline again, owing to the development of immunity.



CHART 4

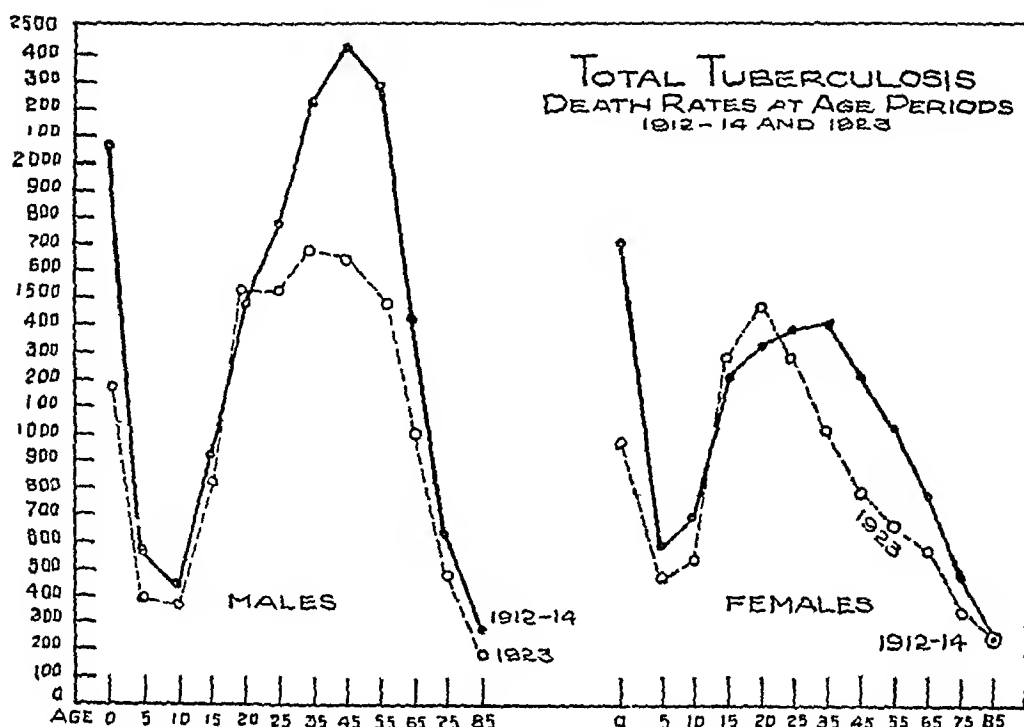
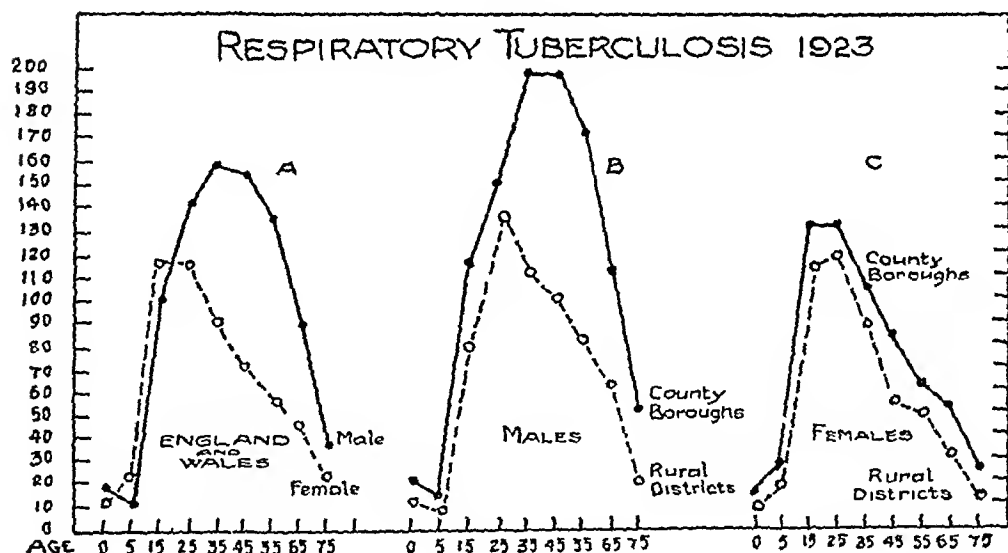


CHART 5



The absence of this curve in India may mean two things —

- (1) Owing to the absence of properly organized children's clinics and hospitals, tuberculous disease and death are not diagnosed and recorded correctly,
- (2) The presence of an immunity in infants and children due to some yet unexplained factors

The former cannot be contradicted. The immunity, if it exists at all, must be non-specific, otherwise the positive cuti-reaction figures would have been higher. In our tuberculin survey in tubercular homes, we have seen that the barrier is only apparent and relative, as 64.5 per cent of such children give a positive reaction. Tuberculous disease is also rare during this period. But we have to leave this question open till we can follow the children of tuberculous mothers for some time more.

*Influence of environment and social conditions on cuti-reaction*

These have already been touched on in the introductory remarks in this paper. We shall touch only on two points here. Though labourers are recruited from the rural tracts, the large aggregation of people in industrial areas gives rise to a high incidence of cuti-reaction, as shown below —

*Mill hands*

*According to age and intensity of reaction*

Age	Intensity of reaction					Total number tested	Per cent positive
	+++	++	+	±	—		
16—20 years	1	6	22	4	19	51	58.0
21—25 ,		11	25	6	18	61	65.5
26—30		14	24	4	10	52	76.0
31—40		7	29	6	10	52	74.5
41—50 „		7	13	2	4	26	82.0
51—60 „		1	5	1	1	8	81.0
TOTAL	1	46	118	23	62	250	70.3

The effect of purdah system (in towns) is manifested by increased cuti-reaction in girls from the time they begin to be confined in their houses, e.g.,

*Percentage of positive cuti-reaction*

Age	Male students	Female students
6—10 years	29.5	30.3
11—15 „	29.7	35.0
16—20 „	29.9	58.0

mortality from lung tuberculosis, which is practically the only lethal form of tuberculosis causing 95 per cent of the mortality, also follows the same lines

CHART 7

AGE INCIDENCE OF PULMONARY TUBERCULOSIS IN BENGAL  
(ON 1300 CLINICAL CASES)

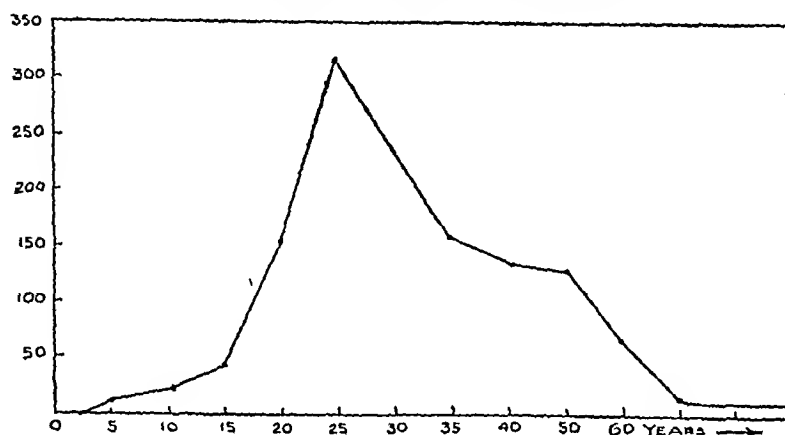
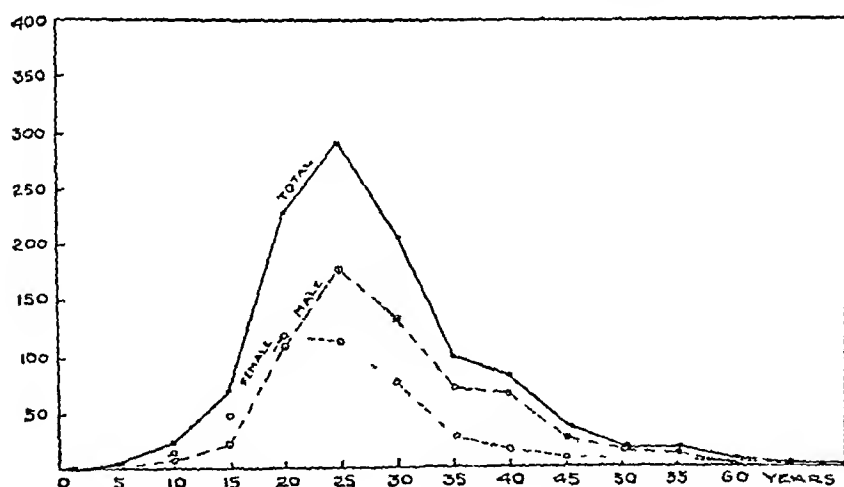


CHART 8

AGE INCIDENCE OF PULMONARY TUBERCULOSIS IN INDIA  
(ON 1,065 SANATORIUM CASES)

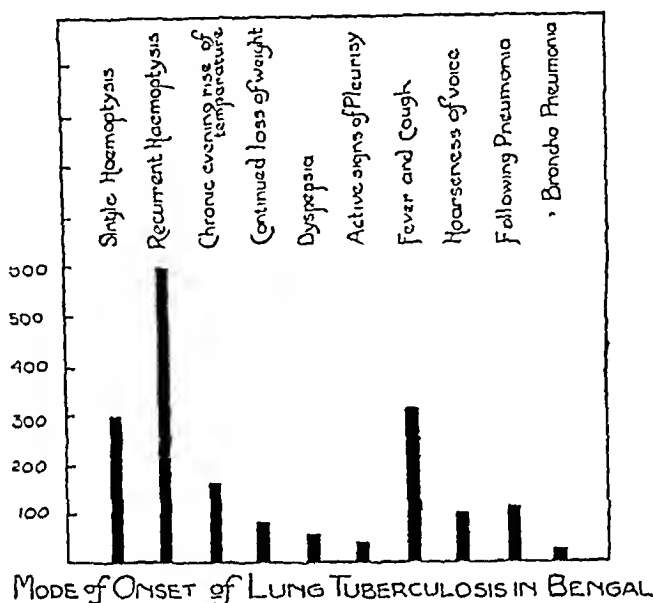


Primary intestinal tuberculosis and tabes mesenterica are most frequent between the ages of 25—35 years. Glandular tuberculosis chiefly affects the cervical region, and less frequently the axillary and rarely the inguinal groups. In children below 10 years, we have failed to find evidences of enlargement of hilar glands, as revealed in skiagraphs, in many cases which showed enormous enlargement and caseation of cervical glands with elevation of temperature.

*The mode of onset of lung tuberculosis*

The mode of onset as gathered from the histories of 1,300 pulmonary cases is depicted graphically in Chart 9. There were 3 cases in this series which had an onset like typhoid fever.

CHART 9



*The part played by bovine tubercle bacilli in human lesions in India*

Tuberculosis in cattle has generally been considered a rare disease in India. Soparkar has found Indian breeds to be more resistant to tubercular infection than English breeds, in spite of their poor physique. Tuberculous udders have not yet been reported. Joshi (7) proved by animal inoculation of several hundreds of samples of cows' milk in Bombay that it was free from tubercle bacilli.

Out of 50 strains of tubercle bacilli isolated from glandular and osteo-articular lesions in man by us, none has yet been found to belong to the bovine type. Laston and Soparkar also obtained similar results in Bombay in 1917. In view of this fact and because milk is generally boiled before taking, the chances of contamination of man by the bovine tubercle bacilli must be considered to be extremely rare in India.

But recent happenings in the Madras Corporation cattleshed (verbally communicated by Prof. Krishnamurthi Ayyar of the Madras Veterinary College), throw some doubt as to the possibility of this state of affairs always existing. Cattle are kept there in crowded sheds away from sunlight. Quite a number of animals have recently succumbed to tuberculosis showing extensive lesions in intestines, mesenteric glands, kidneys, tracheo-bronchial glands and lungs.

It seems that bovine tuberculosis may become more frequent with greater urbanization and confinement of cattle in dark sheds. It is important to know, in this connection whether the relative immunity of Indian cattle is due to their outdoor life. Is it the same thing with young children in India? We have already shown that by confining young girls within houses, the incidence of cuti-reaction distinctly rises.

*The influence of climate on the incidence and types of tuberculous disease*

Climate *per se* seems to have little influence on the incidence of manifest tuberculosis, except that a hot and humid climate has an unfavourable effect on the general health. Tuberculosis has, however, been found to be more common in parts of India, e.g., on the South West coast, where the rainfall is excessive and prolonged. Whether excessive heat and humidity help in converting a potentially immunizing tuberculous infection into tuberculosis disease and of rendering the course of the disease more acute is to be found out, but it is a common experience in the sanatoria of India that excessive heat and humidity have a detrimental effect on the progress of a case.

*Metabolic factors in determining tuberculous conditions in India*

Montel(8) in Cochin China and de Langen in Java(9) thought that the incidence and course of tuberculosis in those countries depended, to some extent, on the low calcium content of soils and water and of cholesterol in the blood of the people of those countries. We collected data on the calcium content of the soils and water of India, through the kind help of the Agricultural Department and compared them with those in England and found that it did not deflect towards the deficiency side in any way, being, on the other hand, actually higher in many parts. The normal calcium content of the blood serum in most young Bengalees varies between 9.2 to 10.1 mg per 100 c.c., with an average of 9.6 mg per 100 c.c. The cholesterol content of the blood in normal individuals in Bengal has been found to vary between 124 to 160 mg per 100 c.c., with an average of 140 mg per 100 c.c. (Mukherjee, *Cal Med Journ* Dec 1928).

*Clinical types of lung tuberculosis*

The types of lesion in the lung will vary to a great extent according to the amount of the infective doses inoculated and re-inoculated, the age, sex and the presence of immunity in an individual. Massive infection is the rule in a majority of cases, as has been stated before. Thus, exudative lesions preponderate in a rural individual in the face of a massive infection.

At the Madras Tuberculosis Institute, 15 per cent of admissions of males and 20 per cent of females are acute cases.

The duration of life, which depends on the infecting dose, the age, the extent and multiplicity of lesions, on the secondary bacterial associations and on the immunity of the individual, is much shorter in India than in Europe. It is shorter

in females than in males. In Europe and America, the average duration of life in the case of lung tuberculosis is from 3 to 10 years, but in India the following ratios hold good in average cases if modern treatment is not undertaken or is not successful—

16—25 years	6 months to 2 years
26—40 years	1 to 3 years or more
Above 40 years	3 to 5 or to 10 years as age advances
Pregnancy and lactation, diabetes, influenza and kala-azar have been found to shorten the course	

Location of lesions	Male	Female
	Per cent	Per cent.
Upper lobe { right	35.4	32.0
	left	18.0
Both apices	6.1	9.0
All over lung	11.8	26.5
Multiple localizations	36.1	35.0

#### *Location of cavities in males and females*

(Verbally communicated by the authorities of the Madnapalle Sanatorium, Madras.)

Male	Cases	Female	Cases
Right side	93	Right side	42
Left side	80	Left side	72
Both sides	93	Both sides	64
TOTAL	266	TOTAL	178

The majority of cases in adults in most parts of India belong to the fibro-caceous type with localization usually in the upper lobes, and less frequently in the upper part of lower lobes.

The lesion seldom remains localized, rapidly spreading to other parts of the lung. The pneumonic type is occasionally met with (2 per cent of cases) in young rural individuals exposed to massive infection. The broncho-pneumonic form is more rarely met with in adults (1 per cent). In about 7 per cent of cases the enlargement of hilar glands, without lung lesions, gives rise to the main symptoms.

#### *Post-mortem evidence*

This is based on 1,000 consecutive post-mortems performed in Calcutta during the last 13 years, 416 cases autopsied at the Madras Medical College during 1927 and 1928 and 113 cases autopsied at the Patna Medical College from 1926-28. The data in Madras and Patna agree in essential particulars with those obtained in Calcutta. We shall therefore give an analysis of Calcutta autopsies in detail.

*Post-mortem evidence (on 1,000 autopsies)*

	Per cent
I Total number of tuberculosis cases—176	
Death due to tuberculosis among medical cases autopsied	12·8
Tuberculosis found complicating other diseases	4·8
Total number of cases which showed well-marked tubercular lesions	17·6
II Primary intestinal ulceration found in	5·1
Secondary intestinal ulceration found in	51·1
Old pleural adhesions found in	72·0
Soft adhesions found in	12·0
Evidence of calcification of old lesions found in (cf 5 per cent in Madras)	31·55
Well-marked caseation present in enlarged hilar glands (cf 50 per cent in Madras)	26·1
General and meningeal tuberculosis in children under 10 years	5·0
III Distribution of pulmonary lesions —	
Upper lobe	47·0
Lower lobe	29·0
Middle lobe of right lung	23·3
Multiple cavities in both lungs	14·0
Extensive involvement of both lungs	62·0
Evidence of miliary tuberculosis	42·0

If we follow Kaufmann's classification the proportion of cases in percentage stands thus —

	Per cent
Total	12·8
Non-fatal	4·8
Latent active (chiefly caseation)	6·1
Latent non-active (chiefly calcified foci)	0·5

Pleural adhesions were found to be very frequent, multiple and extensive

Enlarged bronchial glands, usually 1—2 cm in diameter, were present in most of the fibro-caseous types of lung tuberculosis. The hilar and broncho-pulmonary groups were also involved. A large proportion of the glands (30—50 per cent) showed well marked caseation with little attempt at fibrosis.

The prevailing type of lung tuberculosis was found to be the fibro-caseous form with primary localization in one of the lobes and then rapidly involving other parts. Cavity formation takes place quickly in the involved lung areas, multiple cavities being present in as many as 14 per cent of cases. In a majority of cases between 15—30 years, the cavity walls are not smooth but ragged, caseous and surrounded by caseous and conglomerate tubercles, showing that the degenerative processes are continuous and do not allow time for the development of a wall of reactive granulation tissue around the foci. Out of over 100 cavity walls examined by histological methods, we found well-marked development of connective tissue only in 10 per cent of cases. There is a moderate amount of connective tissue formation in another 10 per cent of cases. The reactive connective tissue proliferation in other cases is very fragmentary and, owing to this poor attempt at localization, the barriers soon break away extending to other parts.

of the lung, showing extensive involvement over both lungs in a large percentage of cases (over 62 per cent), until ultimately the last barrier gives way to an explosion of miliary dissemination (in 42 per cent of cases). Cavity formation is chiefly produced by the liquefaction and sequestration of caseous pneumonic or broncho-pneumonic areas and more rarely of endo- and peri-bronchial caseating foci. The caseous ulcerative process is probably assisted by the presence of secondary organisms which gain entrance through the bronchial tree. Extensions from a cavity have been found to take place both by aspiration and through peri-bronchial and peri-vascular lymphatics. It is thus evident that the exudative changes are a much more prominent feature of lung tuberculosis in India than proliferative or indurative processes which are only found in individuals above 40 years who have been born and brought up in thickly populated urban areas.

Broncho-pneumonia with great enlargement of hilus glands as seen in the infant is a comparatively rare picture (2—3 per cent), having been observed in a few adolescents between 15—20 years of age. In the absence of children's hospitals and of facilities for autopsies on children, it is difficult at the present time to state the details of pulmonary tuberculosis in infants and children in this country.

*Tubercular lesions in other organs (176 cases)*

	Per cent
IV Intestinal ulceration with involvement of mesenteric glands	51.1
Enlargement of mesenteric glands without visible intestinal ulcers	11.3
Intestinal ulceration without visible lung lesions	5.1
Spleen	16.4
Liver	17.0
General peritoneum (miliary)	13.0
Kidneys { Right	9.6
{ Left	7.3
Appendix	3.9
Gall bladder	1.1
Pancreas	1.7
Prostate	0.56
Mouth and pharynx (tonsil, tongue and pharynx)	1.7
Larynx	8.5
Trachea	1.7
Pericardium	4.5
Heart (right auricle)	0.56
Base of brain	4.5
Thyroid	0.56
Tubercular glands other than bronchial	11.9
Abdominal retro-peritoneal	5
Inguinal	3
Axillary	3
Cervical	10

General and meningeal tuberculosis in children under 10 years has been found by Rogers to be 5.0 per cent (*cf.* 62.7 per cent in London).



Out of 416 autopsies in Madras, the distribution of tubercular lesions was found to be as follows —

Lungs	39 cases
Intestines	15 "
Peritoneum	3 "
General miliary	5 "
Spine	2 "
Tabes mesenterica	2 "
	<hr/>
TOTAL	66 cases

Out of 113 autopsies at Patna, 30 cases showed tubercular lesions

### *Pathology of lung tuberculosis in India*

Infection with tubercle bacilli depends on several factors—(1) the virulence of the bacilli introduced into the body, (2) the number of bacilli simultaneously introduced, (3) repeated infection at shorter or longer intervals, (4) the sensitiveness of the individual, and (5) the path of infection, i.e., whether inoculated through susceptible or non-susceptible tissues

As a result of extensive work by several workers, we are now realizing that the disease picture in tuberculosis, especially of the lungs, can be explained in terms of the immunological response of the body. Ranke's conception seems to us to fit in with most of the problems. The primary focus after infection in inhalation tuberculosis in a non-immunized individual is a small exudative miliary broncho-pneumonia about the size of a hempseed near the pleura in the upper lobe below the clavicle. The draining lymph nodes are always involved with caseation and surrounded by a fibrous capsule. This is quickly followed by productive lesions surrounding the first focus. The primary changes clear up rapidly in the majority of cases. From 2 to 3 weeks after this, a state of allergy or hyper-sensitiveness of the cells of the body develops as a response to the primary infection. Allergy might be called the reaction of the tissues against tuberculo-protein, while immunity is the reaction against the bacilli. The larger the amount of inoculation that an individual is able to overcome, the higher will be his ability to 'react allergically'.

In the study of the pathology of tuberculosis, tuberculin surveys are a very important aid in determining the state of immunity of a race. The percentage of positive skin tests will be higher where chronic pulmonary tuberculosis is the prevailing type, it is low where tuberculosis is rarer but more severe and acute. In the *second stage or stage of allergy*, which exists so long as viable bacilli exist in unhealed foci or re-infections take place from outside, successive re-inoculations or re-infections guide the whole process. Proliferative or exudative reactions predominate according to the dose and virulence of the bacilli, the amount of tuberculo-protein diffused into the tissues and the state of allergy and resistance of the host. If the dose of the re-infecting bacilli is small, the exudative changes subside quickly giving place to productive lesions. Caseation and

necrosis occur in tissues due to allergic inflammatory reaction. The inoculation of massive doses at frequent intervals in the presence of a low specific reaction would account for a rapid caseation with marked loss of tissue and cavity formation in the course of the disease. This prevents the formation of adequate walling off of the focus by the production of fibrous tissue by peri-focal reticular cells in response to the stimulation of tuberculo-protein and thus furnishes a leak for the re-infecting bacilli to extend the process. Different organs and even different parts of the same organ may be in a different state of allergy. In this stage the changes are manifested by exudative reactions at sites of metastases and marked peri-focal inflammation frequently followed by caseation. This may lead to hematogenous metastasis in bones, joints, lymph-nodes, kidneys, skin, etc., or end in the malignant form of generalized miliary tuberculosis. This is followed by the *third or tertiary stage* and its transition forms, in which the state of hyper-sensitiveness subsides and the exudative reactions become less violent due to the development of a relative immunity. Productive reactions are more frequent and localized without involvement of lymph-nodes. Exudative changes take place when the immunity breaks down towards the last stages. Metastases by lymph and blood become less and less frequent but contact growth and broncho-genic extension within the organ may occur as before.

The power to restrain the multiplication of tubercle bacilli and to wall off tubercular lesions effectively by the growth of connective tissue is the manifestation of a degree of immunization which is only attained in the course of some years after infection. The lung tuberculosis of the adult type thus differs considerably from that in childhood.

Besides these immunological factors, age has an important bearing on the type and the course of the disease. In young children the lymphatic channels are wide open and allow the passage of bacilli into the lymph stream and circulation easily. This, as well as the non-development of allergy, account for the high peak of mortality in infancy and early childhood in Western countries. As age increases the lymphoid tissue increases in amount and impedes the flow of lymph and thus mechanically obstructs the passage of tubercle bacilli. The fall in mortality figures between childhood and adolescence is due to the establishment of allergy. The allergy has decreased by the time adolescence is reached while the lymphatic defence still remains immature. From this period onwards the mortality curve begins to rise owing to various conditions of lowered vitality and to dosage and frequency of re-infections. The final fall of the curve occurs after the 45th year in Western countries and 35th year in India due both to the increase of lymphatic resistance and to the development of immunity.

Let us try to interpret the pathologic lesions found here in terms of this conception.

The first thing which strikes one is the comparative low morbidity as well as mortality from tuberculosis in childhood up to 10 years, in very marked contrast to the facts in Europe. The low incidence (5 per cent) of general and meningeal tuberculosis is in striking contrast with the high figure of 62.7 per

cent in London Tuberculous meningitis occurs in only 1 per cent of the cases admitted into the children's ward of the Medical College hospitals in Calcutta The only forms which occur with any frequency during this period are the glandular and osteo-articular tuberculosis, which are, however, more frequent here than in Europe This low incidence of tuberculosis in infancy and childhood in presence of a low degree of bacillization has always been a puzzling phenomenon to us We shall look forward to more definite data when children's autopsies are more frequently carried out here When infants and children are exposed to heavy infection by being nursed by tuberculous mothers, we found them to live and increase in weight up to the period of observation utilized by us (viz, 3 years) Most of them show, however, a large number of greatly enlarged and caseating cervical glands without any apparent increase in size of the hilar glands as revealed by the X-rays We have shown that these are benign forms of tuberculosis caused by the human type of tubercle bacillus Whether the non-involvement of lung tissues and the rarity of dissemination through the circulation is an indication of a degree of immunity possessed by such children is difficult to say The survey and observation of tubercular homes is being continued and it will take some time to arrive at more definite conclusions regarding this question

Two important facts must be remembered in studying the pathology of the disease in India —

(1) The comparatively low degree of bacillization of the general population which means that a major part of the population is imperfectly immunized,

(2) That massive infection is the rule, whether initial or repeated, owing to certain social customs and habits already mentioned As evidences of this statement, we may say that we very often find a fair number of tubercle bacilli even in direct smears of the pus from glands and other surgical lesions A direct culture has been obtained in 14 out of 50 strains isolated by us The comparative frequency of primary intestinal tuberculosis presumably from swallowing heavily contaminated food and drink, and of a caseous involvement and limitation to glands of the cervical groups points to the frequency of infection through mouth and pharynx The chances of contracting the disease outside dwelling-houses in places exposed to sunlight is very small, owing to the quickly lethal action of the sun's rays in this country for most part of the year [Uki and Basu, 1927(11)], Soparkar(12) The extremely careless method of living in India makes the chances of chronic vaccination through inhalation or ingestion of alternated bacilli very small

The incidence of tuberculo-allergy rises rapidly after the 15th year when the diffusion of population begins to be more common We also find the different forms of tuberculosis in the largest numbers between this and the 40th year

Systematic examination of lung tissues obtained from several provinces has shown that cavities in the lungs very rarely show smooth walls showing well-developed fibrous tissue The walls are usually ragged and caseous Histological examination of sections stained with hæmatoxylin and eosin and by Van Gieson's method show, in most cases, a poorly-developed connective tissue formation

Exudative changes are more frequent than proliferative reactions. Involvement of multiple areas of the lungs occurs early and show the same changes.

Almost all the evidence obtained point to the changes and the course of the disease being acute as compared with those in Europe, due to a partially immunized soil being invaded by massive infection. The immunity of the well-immunized individual or the immunity developed by minute doses received at infrequent intervals as seen in more thoroughly urbanized Western countries, we see here only in individuals above 40 and in persons brought up in thickly populated urban areas. The whole pathological anatomy of the lungs points to the changes being due to massive infection on an imperfectly immunized soil. The evolution of the form of the disease here seems to depend more on the dose of infection (=massive infection) than on the lack of immunity of the individual. Secondary bacterial association in 'open' lung tuberculosis are more frequent and richer here than in Europe. We have shown in another paper (13) that, apart from ulcerative processes, the association of secondary organisms increases the virulence of tubercular bacilli. These factors probably account for a more rapid course of the disease after the lungs are once involved. The more acute course in females is probably due to their close and sedentary life and to early marriage and child-bearing.

The glandulo-pulmonary or infantile type as seen among the absolutely non-immunized races like the Africans, are rarely met with on the autopsy table.

The following comparative figures will further show that the state of specific immunity against tubercular infection attained by the major part of the population of India seems to be intermediate between that attained by the Africans and Europeans and that India and China probably stand on the same level.

*Some comparative figures showing types of lesions found*

	Calcutta Per cent	Edinburgh (Todd) (14) Per cent	London (Fowler) (15) Per cent
<i>I Necropsies on hospital cases</i>			
Total tubercular lesions	17.6	69.0	
Death due to tuberculosis	12.8	3/386 cases	
Calcereous lesions in lungs (Cf Madras)	31.55 5.0	64.0	
Latent non-active lesions	"	9.0	9.0 (obsolete)
Caseation in bronchial glands (Cf Madras)	26.1 50.0	Very few	9.5
Spleen	16.4	2/386 cases	2.7
Liver	17.0	Not noted	1.6
General peritoneum	13.0	"	4.1
Pericardium	4.5	"	Rare
Larynx	8.5	"	47.0

*II Necropsies on an apparently healthy population*

	Calcutta Per cent	British Soldiers in Etaples (Rose-Bradford, 1920) (16) Per cent
Pleural adhesions	34.4	
Tubercular lesions in lungs	6.6	12.0
Latent active lesions	6.0	?
Latent non-active lesions	0.6	5.0 (or 75 of total tuberculosis)

*III Comparative incidence and deaths among B. E. Force in France in 1918  
(Cummings, 1921) (17)*

	Annual case incidence per 10,000 of annual average strength	Annual deaths per 10,000 of annual average strength	Proportion
South African Native Labour Corps	186	167	9/10ths
Indian Labour Corps	142	53	Nearly 1/3rd
Chinese Labour Corps	36	12	1/3rd
British troops in France and Belgium	10	0.5	1/20th

Only two roentgenographs (Plate LX) of the chest are reproduced to show characteristic changes—Fig 1 showing lesions in a middle-aged villager living away from rail routes and in a family where several had died of lung tuberculosis. Fig 2 shows the lesions in a young medical student who had come to Calcutta only recently for studies and who had a hæmoptysis only, being an ambulant afebrile case.

## CONCLUSIONS

1 Though the Indian cities are fairly bacillized, the general mass of population in India is imperfectly immunized against tubercular infection. The average bacillization is yet far below that of Western countries. The distribution has been mapped out and the causes ascertained.

2 The absence of the peak of tuberculosis mortality in infancy and early childhood in presence of a low degree of bacillization is puzzling and needs further investigation.

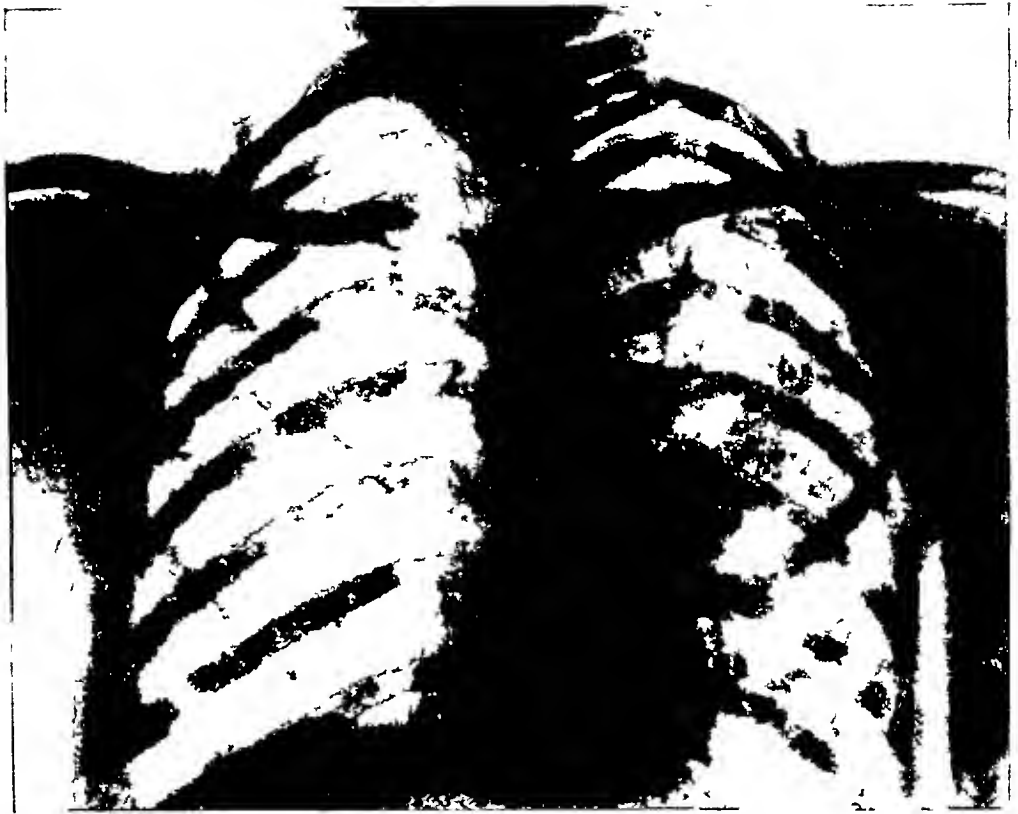


Fig 1





3 Massive infection operates in a majority of cases of tuberculous disease and this factor acting on an imperfectly immunized soil determines the type of lung lesions, their course, their prognosis and even their treatment

4 As the mass of population gets more perfectly bacillized (or urbanized), the tuberculous lesions will be more chronic in course resembling those in Europe. The disease incidence will not diminish even then unless the chances of massive infection are lessened

5 The more acute course of the disease in India is partly due, apart from the question of massive infection operating on an imperfectly immunized soil, to the more frequent and richer secondary bacterial associations in 'open' pulmonary cases and to the increase of virulence of such association

6 Glandular and osteo-articular tuberculosis in India are to be considered as benign forms due to the human type of tubercle bacillus

7 An attempt has been made to interpret the pathology of the disease in India in terms of allergic manifestation

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# THE ROLE OF SECONDARY BACTERIAL FLORA IN PULMONARY TUBERCULOSIS

BY

A C UKIL,

*Professor of Bacteriology, National Medical Institute and Officer-in-charge,  
Tuberculosis Research Indian Research Fund Association Calcutta*

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## INTRODUCTION

THE present work arose out of our studies on the 'Epidemiology of Tuberculosis in India'. It has been found that pulmonary tuberculosis is a much more acute disease and the duration of life shorter in most parts of India than in Europe or America. Though our epidemiological studies furnished sufficient data to account for this, the further question arose whether the secondary bacterial flora in 'open' pulmonary tuberculosis was in any way responsible for influencing the pathology or shortening the life of the cases in this tropical country.

In this paper we shall not consider the part played by acute infections like influenza, pneumonia, eruptive fevers, etc., or by chronic infections like syphilis, malaria, kala-azar, leprosy, fungoid diseases, etc., in influencing the march of lesions. We shall only consider the part played by secondary bacteria in 'open' pulmonary tuberculosis, i.e., when a lesion in the lung opens into a bronchial tube and thus communicates with the exterior. It is well known that so long as a tubercular lesion in the lung remains 'closed,' the tubercle bacillus alone accounts for caseation, cavitation and hectic fever. These occur also in extra-pulmonary localizations like psoas abscess or renal tuberculosis, which are absolutely closed lesions.

The varying clinical manifestations and morbid anatomy of the disease presenting a complexity of signs and symptoms have been the subject-matter of enquiry by various workers since Koch found the occurrence of secondary bacteria in the walls of tuberculous cavities in the lung in 1884 and since Koch and Kitasato tried to eliminate saprophytic bacteria by repeated washings of the sputum seven years later. Opinion is sharply divided in this matter, there being two schools of thought. One school consisting of Babes, Spengler, Sehabed and

others think that the lungs are sooner or later invaded by secondary organisms and that a considerable proportion of the patient's symptoms is attributable to secondary infection, specially hectic fever and aggravation of the general condition. They, therefore, advocate the use of mixed vaccines in combating secondary invasions. The other school, consisting of Sergo, Karl Thue, Halbron, Bezançon, Biros, Chevalley (1923) and others(1), think that, in a majority of cases, the tubercle bacillus alone is the dominating factor in producing cavities, temperature and toxic symptoms, and that secondary bacteria, when found, come from contamination during the passage of the sputum through the air passage and mouth in the process of being coughed out. The latter school think that, in a majority of cases, the secondary bacteria are not associated with the tubercle bacillus in the anatomo-pathological processes and that the findings of previous workers in the line were vitiated by the fact that the sputum after collection was either not washed through several changes of saline or not examined within 3 hours after leaving the body. Haemoculture has given negative results with them, except towards the last stages. They think that, when secondary bacteria are present, it is an evidence of bronchial engorgement and inflammation or of pharyngeal catarrh.

Inman(2) of the Brompton Hospital in London made a study of the subject in 1912 and his conclusions were as follows —

- (1) In nearly every case of 'open' tuberculosis of the lungs, the tubercle bacillus is the predominant infecting agent.
- (2) Determinations of the opsonic index show that secondary infections do occur in practically all cases of 'resting febrile' cases and, in a fair number of cases of 'ambulant febrile' cases, the actual proportions being —

	Febrile cases Per cent	Afebrile cases Per cent
Infection by T B and secondary infection	87.5	54.1
Infection by T B alone	12.5	41.6

- (3) The temperature chart alone cannot determine the presence or absence of a secondary infection.

Courmont and Boissel(3) carried out a large series of experiments at Lyons (France) and published their results in 1924. They found that while secondary infections were much less frequent than was formerly supposed they were present in a considerable proportion, viz., 20 per cent, while in the remaining 80 per cent the tubercle bacillus alone was sufficient to produce every form of anatomo-pathological lesion of the lungs and all the clinical varieties of the disease. They thought that these microbial associations affected the prognosis of the cases.

Efforts have been made from time to time to ascertain the bacterial flora in cavities post-mortem. Wassermann and Cornil combined a bacteriological study of the sputum with a subsequent search for micro-organisms recovered from the

cavities post-mortem. The results in their hands on the whole corresponded Veillon and Replat (1912)(4) made a bacteriological investigation of material from cavities obtained post-mortem using both aerobic and anaerobic culture methods. They concluded that in a majority of cases of open pulmonary tuberculosis secondary organisms were present and that in rare cases Koch's bacillus alone was present. They isolated various aerobes and anaerobes from the contents of cavities and thought that the secondary aerobic organisms did not influence very much the appearance of sputum whereas the anaerobic organisms, when present, were often responsible for the fidity of the sputum or gangrenous condition of the lungs.

In our study we have utilized only the sputum of open pulmonary cases and have refrained from making a comparative study of the material from cavities, for the reason that there is no cool chamber for preserving dead bodies in hospitals in Calcutta and because post-mortems often have to be done long after death. Such materials, even if studied, would not represent the actual bacterial flora in cavity cases. We reserve this for a comparative study later on.

We have followed a careful technique which will be detailed below.

#### TECHNIQUE EMPLOYED

*Number of cases studied*—In all 100 cases have been studied, but the data from 40 cases only are included in this paper, as being those in which an unimpeachable technique has been followed. All the cases belonged to the Tuberculosis Ward of the Medical College Hospitals, Calcutta.

*Collection of sputum*—The patients were asked to clean their mouths thoroughly by rinsing and then to cough out from the inner air passages into sterile glass tubes in presence of a worker under this enquiry. The materials were immediately brought to the laboratory and the study begun.

*Procedure of examination*—A yellow particle of sputum was taken and washed successively through several changes of physiological saline in test tubes each containing 10 c.c. Washing was continued so long as bubbles of air came out of the particles of sputum.

A good deal of divergence of opinion exists as to the best method of getting rid of the saprophytic organisms in the bronchial and nasopharyngeal passages. It was, therefore, thought necessary to make a comparative study before getting on to a fixed method of investigation.

Smears as well as cultures of each sample of sputum were made before washing and after washing 3, 4, 5, 8 and 10 times in sterile physiological saline. The following conclusions were arrived at—

- (1) The number of all micro-organisms diminishes (in smears) by washing, the more they are washed the more they diminish in number. But the secondary organisms diminish much more quickly in number than the tubercle bacillus. Repeated washings diminish the number of tubercle bacilli by only 15 per cent, whereas 50–60 per cent of the

secondary organisms are washed out by 4 washings, 60—80 per cent by 5 washings and 80—90 per cent by 8—10 washings

- (2) The numerical proportions were noticeable to a less degree in cultures (aerobic) Even where no secondary organisms were detected in stained smears, a fair number of micro-organisms appeared on the surface of the culture media
- (3) The cellular elements are much reduced in the process of washing (by 1|3rd), but the relative proportion of leucocytes in differential count remains the same

About 2|3rds of the samples of sputum included in this study were washed 4 times and the rest 5—8—10 times before the findings were recorded

*Examination of smears*—Three thin smears of the washed out sputum were made and stained by Gram's method, Ziehl-Neelsen's method and Leishman's stain respectively

The varieties and relative proportion of micro-organisms and of cellular exudate were noted

*Culture (aerobic)*—A loopful of the cleaned sputum was taken and planted on to Loewenthal's medium, blood agar, nutrient agar media and to lactic acid agar (0.3 c.c. of 5 per cent lactic acid to each tube) if yeast cells were present Colonies were next identified in the usual course

*Culture (anaerobic)*—A washed particle of sputum was emulsified in nutrient broth and transferred on to glucose broth, egg broth and Veillon's deep agar media Oxygen was abstracted from the liquid media by vacuum Obligatory anaerobic colonies were fished out and purified by a modification of Veillon's technique Pure colonies were cultured in glucose broth media and various quantities were inoculated into guinea-pigs to determine their pathogenicity They were cultured in egg broth to find out if they possessed any proteolytic action on egg-albumin

*Animal inoculation*—Guinea-pigs were inoculated with emulsified unwashed sputum intramuscularly to find out the action of the sum-total of bacterial associations in the whole sputum Guinea-pigs were also inoculated intramuscularly and subcutaneously with each strain of aerobe and anaerobe isolated singly as well as in various combinations with each other and with the tubercle bacillus to study the action of microbial associations

## EVIDENCE OBTAINED

### *I Differential count of leucocytes in sputum smears*

A differential count of leucocytes in the sputum was made to see if there was any relationship between the anatomo-pathological lesion, signs and symptoms and the count All the cases were 'resting febrile' and 'open' pulmonary The duration of illness of the cases varied from 2 months to 1½ years, except in 3 cases who had been struggling for 3 or 4 years

The following list speaks for itself. It will be found that no information regarding the type or severity of a lesion can be gathered from such counts

	Minimum and maximum temperature (in degrees Fahrenheit)	Nature and extent of lesion	DIFFERENTIAL COUNT OF LEUCOCYTES PER CENT			
			Polymorpho-nuclear	Small mono-nuclear	Large mono-nuclear	Eosinophiles
1	99—101	Consolidation—upper half of right lung	72	28		
2	99—100	Cavity—left base	30	70		
3	98.4—100	Consolidation—right apex	10	90		
4	98.4—101	Consolidation—right apex	24	74		
5	98.4—102	Cavity—right apex	56	44		
6	100—104	Consolidation—whole of right lung	98	2		
7	99—101	Consolidation—right base	32	68		
8	98—100	Consolidation—right base	96	4		
9	98—100	Consolidation—left base	80	16	4	
10	99—101	Consolidation—both apices	80	20		
11	97.5—102	Consolidation—both apices	68	24	8	
12	97.5—100	Consolidation—left apex	60	36	4	
13	97.5—100	Consolidation—both apices	60	36	4	
14	99—102	Cavity—right apex	65	31	4	
15	99—102	Consolidation—both apices	65	30	5	
16	99.5—103	Cavity—left apex and base	36	56	4	4
17	99—101	Consolidation of whole of back of left lung	44	40		16
18	97—101	Consolidation—right upper lobe	60	40		
19	98.4—100	Cavity—right apex	36	60	4	
20	98.4—101	Consolidation—left apex and left base	76	20	4	
21	97—102	Consolidation—multiple areas all over left lung	64	30	6	
22	99—101	Multiple areas of consolidation—right lung	80	15	5	

	Minimum and maximum temperature (in degrees Fahrenheit)	Nature and extent of lesion	DIFFERENTIAL COUNT OF LEUCOCYTES PER CENT			
			Polymorpho- nuclear	Small mono- nuclear	Large mono- nuclear	Eosinophiles
23	100—103	Areas of consolidation—both lungs	34	65	1	
24	98.4—101	Multiple areas of consolidation—both lungs	42	56	12	
25	97—99	Cavity—right apex	90	10		
26	99—102	Multiple areas of consolidation—both lungs	80	20		
27	97—99	Consolidation—right apex	68	28	4	
28	98.5—100.5	Consolidation—right apex	50	45	5	
29	98.4—100	Consolidation—right apex	64	34	2	
30	99—101	Consolidation—both apices	80	20		
31	99—102	Multiple areas of consolidation—both lungs	20	80		
32	100—103	Cavity—both apices	20	80		
33	99—102	Cavity—upper left lobe	36	56	4	4
34	97—101	Consolidation—left lower lobe	68	28	4	
35	97—101	Consolidation—right base Pleuritis—left base	68	32		
36	97—99.5	Consolidation—left lower lobe	65	30	5	
37	98.4—100	Consolidation—left base	80	16	4	
38	97—102	Consolidation—both apices	68	24	8	
39	97—100	Consolidation—left apex	60	36	4	
40	98.4—101	Multiple areas of consolidation—both lungs	80	18	2	

II *Micro-organisms present in smears*

Besides tubercle bacilli, smears showed a varying number of the following organisms in order of frequency —

*Streptococcus*

*Micrococcus catarrhalis*

*Staphylococcus aureus*

*Micrococcus tetragenus*

Influenza bacillus

Yeast cells

*B. coli*

*Pneumococcus*

*B. pseudodiphtheria*

*B. Friedlander*

Fusiform bacilli and spirochaetes (5 per cent of cases)

Gram positive non-acid-fast bacilli which could not be recovered on culture  
(50 per cent of cases)

III *Micro-organisms recovered by aerobic culture*

	Per cent of cases
<i>Streptococcus non-haemolyticus</i>	40
Do <i>haemolyticus</i>	40
Do <i>viridans</i>	15
<i>Staphylococcus aureus</i>	7.5
<i>Pneumococcus</i>	7.5
Yeast cells	7.5
<i>Micrococcus catarrhalis</i>	5.0
Organisms of the <i>B. coli</i> group	3.7
Diphtheroid bacilli	3.7
<i>Micrococcus tetragenus</i>	3.5
Pfeiffer's bacillus	3.5
<i>B. Friedlander</i>	2.0

IV *Micro-organisms isolated by anaerobic culture*

Obligatory anaerobes were isolated in 11 cases, of which *Streptococcus anaerobius* was present in 2 cases and the rest were cocci in clumps which behaved



rather loosely towards Gram stain. Some of the latter agreed with the description of *Streptococcus parvulus* of Veillon and Repaci showing small fine ovoid diplococcus or small chains arranged side by side or in clumps. Such organisms have previously been described by Lewkowicz (in the mouth of the new born), by Lippmann (in biliary infections) and by Jaumin (in puerperal infections). The colonies are shy to grow, taking 3 to 5 days for a good growth. Subcultures are easy to make in recent cultures but are difficult in old cultures, for the reason that they require frequent subcultures to keep them living. They did not show any proteolytic properties. The anaerobic flora in non-putrid pulmonary tuberculosis in this country does not seem to be so rich and varied as in Europe.

The naked eye appearance of the colonies in deep agar and the microscopic appearance of the organisms are depicted in the microphotographs reproduced below —

#### PATHOGENICITY EXPERIMENTS

##### *Direct subcutaneous inoculation of the sputum*

In each case a fairly opaque emulsion of the sputum was made in normal saline and 1 c.c. was inoculated subcutaneously into two guinea-pigs. This was done with a view to find out the action of all the microbial associations as they existed in fresh sputum. The animals were observed from day to day and the lesions were noted. On death, an autopsy was made as well as a hæmoculture. Hæmoculture was invariably negative—the results were as follows —

	Per cent
Considerable swelling and other signs of inflammation	10.4
Do do and death	7.8
Do do and abscess formation and death	5.2
Moderate swelling	28.6
Do do and abscess formation	2.6
Do do do and death	2.6
Slight swelling	23.7
Do do and abscess formation	2.6
Do do do and death	2.6
Do do death	5.2
No lesions	5.2

The inflammation lasted for from 3 days to 3 weeks, the animals dying later on from tuberculous lesions.

## Pathogenicity of aerobic organisms isolated

Usually a 24 hours' broth culture of the organisms was inoculated intramuscularly into the thigh muscles of the guinea-pig and the swelling or other lesions were noted from day to day

	Dose inoculated in c.c.	Lesion	Dose inoculated in c.c.	Lesion
<i>Streptococcus haemolyticus</i>	0.5	Slight swelling subsiding in 2 to 4 days	2.0	A moderate degree of swelling, subsiding in 2 to 4 days
<i>Streptococcus non-haemolyticus</i>	0.5	Do	2.0	Do
<i>Streptococcus viridans</i>	0.5	Do	2.0	Do
<i>Micrococcus catarrhalis</i>	0.5	Nil	2.0	Very slight swelling
<i>Pneumococcus</i>	0.5	Nil	1.0	Swelling ++ subsided in 5 days
<i>Staphylococcus aureus</i>	0.5	Nil	2.0	Slight swelling subsided in 4 days
Pfeiffer's bacillus	0.25	Nil	0.5	Experiment repeated 4 times. All the guinea-pigs died. Subperitoneal hemorrhages, exudation of sero-sanguinous fluid in peritoneal cavity, kidneys congested. Site of inoculation moderately swollen. Hemoculture negative.
<i>B. coli</i>	0.5	Swelling ++ developed into an abscess in 7 days	1.0	Edema around site of inoculation. Swelling ++. Death in 6 cases. <i>B. coli</i> isolated from hemoculture.
<i>B. proteolyticus</i>	0.5	Edematous swelling ++ subsided in 6 days	1.0	Edematous swelling ++. animal died in 48 hours.
Diphtheroid bacilli	0.5	Nil	2.0	Nil
<i>B. Friedlander</i>	0.5	Nil	2.0	Slight swelling subsided in 4 days

*Pathogenicity of anaerobic organisms isolated*

A 24 hours' culture of the pure organisms in glucose broth was inoculated intramuscularly into 2 guinea-pigs. Lesions were noted in the shape of swelling and tumefaction of the thigh and were classified into 3 categories—slight (+), moderate (++) and marked (+++) swelling. None of the animals died but those which suffered from considerable swelling and œdema remained ill and inactive for a variable number of days. The animals were observed from day to day and the lesions noted.

The details are given in the following table —

Description of strain	Dose inoculated in c c	Degree of inflammation	Inflammation subsided in	Dose inoculated in c c	Degree of inflammation	Inflammation subsided in
Cst	10	—		50	+++	12 days
8t	10	+	2 days	50	+++	15 "
IIL	10	++	4 "	50	++	9 "
12t	10	++	4 "	50	++	7 "
15t	10	+	2 "	50	++	5 "
16P	10	++	7 "	50	+++	9 "
17P	10	+	5 "	50	++	5 "
18t	10	++	4 "	50	+++	6 "
20t	10	+	3 "	50	+	4 "
23t	10	+	3 "	50	+	3 "
21t	10	+	3 "	50	+	3 "

*Association experiments (in vivo)*

No microbe is indifferently present in a lesion. It must either assist or retard the action of one or more of the other microbes present in the lesion.

The end results or symptoms and signs in a case depend on a resultant action of all the microbes present in a lesion. The severity of a case depends on the quality and quantity of the microbes, on the intensity of their bacterial interactions, on the local and general predisposing factors present and on the individual susceptibility of the animal.

To find out whether the micro-organisms isolated on culture after a thorough washing of the sputum could in any way, by their interactions, influence the lesions produced in guinea-pigs separately as well as in presence of the tubercle bacillus, we performed three series of experiments —

- I Association between different varieties of aerobes
- II Association between aerobes and anaerobes
- III Association between aerobes and anaerobes and the tubercle bacillus

*I Association between aerobic bacteria isolated from the washed sputum of cavity cases*

A 24 hours' culture in glucose (1 per cent) broth was employed throughout the experiments. The doses were varied by suitable permutations and combinations, employing a small dose of one microbe and a bigger dose of the other and vice versa. The inoculations were given subcutaneously into guinea-pigs of approximately the same weight, the lesions were observed from day to day and weekly weights were taken, keeping some uninoculated animals as controls at the same time.

	Microbic association	Dose in c c	Lesion
I	Streptococcus (hemolyticus)	0.5	Slight swelling subsiding in 3 days
	+ Streptococcus (non hemolyticus)	2.0	
II	Streptococcus (hemolyticus)	2.0	" " " " 3 "
	+ Streptococcus (non hemolyticus)	0.5	
III	Streptococcus (hemolyticus)	0.5	" " " " 3 "
	+ Streptococcus (viridans)	0.5	
IV	Streptococcus (hemolyticus)	0.5	" " " " 3 "
	+ Streptococcus (viridans)	2.0	
V	Streptococcus (non-hemolyticus)	1.0	" " " " 4 "
	+ Streptococcus (viridans)	1.0	
VI	Streptococcus (hemolyticus)	0.5	" " " " 4 "
	+ Streptococcus (non-hemolyticus)	0.5	
	+ Streptococcus (viridans)	0.5	" " " " 3 "
VII	Streptococcus (hemolyticus)	2.0	
	+ Micrococcus catarrhalis	0.5	" " " " 3 "
VIII	Streptococcus (hemolyticus)	0.5	
	+ Micrococcus catarrhalis	2.0	" " " " 3 "
IX	Streptococcus (hemolyticus)	2.0	
	+ Pneumococcus	0.5	Moderate " " " 5 "
X	Streptococcus (hemolyticus)	0.5	
	+ Pneumococcus	2.0	Slight " " " 4 "
XI	Streptococcus (hemolyticus)	2.0	
	+ Staphylococcus aureus	0.5	" " " " 4 "
XII	Streptococcus (hemolyticus)	0.5	
	+ Staphylococcus aureus	2.0	" " " " 4 "
XIII	Pneumococcus	2.0	
	+ Staphylococcus aureus	0.5	" " " " 4 "
XIV	Pneumococcus	0.5	
	+ Staphylococcus aureus	2.0	" " " " 4 "
XV	Streptococcus (hemolyticus)	0.5	
	+ Pneumococcus	0.5	" " " " 4 "
	+ Staphylococcus aureus	0.5	

And so on with the other microbes isolated

Controls were kept by the inoculation of single organisms also

Besides the local inflammation, no animal died or lost much weight

*II Association between aerobes and anaerobes*

In this series of experiments, the aerobic bacteria were inoculated intramuscularly, both singly as well as in different combinations to as con'

Single aerobes as well as multiple aerobic combinations were mixed with each variety of anaerobe isolated during our study. The lesions were noted from day to day and weekly weights recorded as usual. Experiments were repeated in case of the least doubt arising as to the result of an experiment.

Space will not permit us to quote all the experiments in this series. A typical set, when quoted, would be as follows —

	Microbes inoculated	Dose inoculated in c c	Lesions			REMARKS
			Slight	swelling	subsiding in 4 days	
I	Streptococcus	2.0				Aerobic control
	"	0.5	"	"	4 "	"
II	<del>Staphylococcus aureus</del>	0.5	"	"	4 "	"
	"	2.0	"	"	4 "	"
III	Streptococcus	2.0				Aerobic association
	+ Staphylococcus aureus	0.5	"	"	4 "	
	Streptococcus	0.5	"	"		
	+ Staphylococcus aureus	2.0				Anaerobe control
IV	( Anaerobe Strain ) 8t	5.0	Considerable swelling	subsiding in 5 days		
	"	1.0	Slight	"	2 "	
V	Streptococcus	2.0+8t—0.5	Moderate	"	7 "	Mono-aerobe + anaerobe
	"	0.5+8t—1.0	"	"	7 "	
VI	Staphylococcus aureus	0.5+8t—1.0	"	"	7 "	
	"	2.0+8t—0.5	"	"	7 "	"
VII	Streptococcus	2.0				Polyaerobe + anaerobe
	+ Staphylococcus aureus	2.0	"	"	10 "	
	+ 8t—0.5 c c	0.5	"	"		
	Streptococcus	0.5				"
	+ Staphylococcus aureus	0.5	"	"	10 "	
	+ 8t	1.0	"	"		

Similar combinations were made with other aerobic organisms isolated, such as *Pneumococcus*, *B. coli*, etc.

It will be seen that in the association experiments with anaerobes, a smaller quantity of anaerobic culture was taken than employed in the control experiment with the anaerobe alone, to see if, when no lesions are produced by such a small dose of the anaerobes, the association with aerobic bacteria enhances the lesions.

### Results

As a result of the association between aerobes and anaerobes, the reaction, i.e., swelling and tumefaction of the inoculated region was augmented in 5 cases, diminished in 3 cases, unchanged in 3 cases.

### III Association of aerobes anaerobes and the tubercle bacillus

Before performing the association experiments, a *control experiment* was done by inoculating 12 guinea-pigs, weighing from 275 to 300 grammes, subcutaneously with a strain of the tubercle bacillus, labelled as strain '4 S,' isolated from sputum, 0.01 mg. of which killed the animals between the 41st to 45th day. On autopsy, the inguinal glands were found enlarged and caseous and the spleen studded with tubercles.

In all autopsied animals, smears were made from the inguinal and pelvic glands, spleen and other organs showing tubercles with the naked eye and examined under the microscope after staining by Ziehl-Neelsen method. A hæmoculture was invariably made from heart blood, to exclude accidental death or death from other infections. The general appearance of the animals was noted and the weights recorded every week.

For the association experiments, 2 animals were inoculated with the same doses and in case of any doubt such experiments were repeated more than once. Animals weighing between 275 to 300 grammes were employed throughout the experiments.

One of the experiments is quoted below for illustration —

Tubercles were found in autopsied guinea-pigs as early as 15 days and as late as 40 days after inoculation. The average period of duration of life of these guinea-pigs was 23 days.

Seventy per cent of the animals employed in the association experiments died within 3 weeks showing rapid loss of weight, loss of hairs, asthenia and anorexia.

The twelve control animals inoculated with the tubercle bacillus did not show such a rapid emaciation and loss of hairs.

### Discussion

One great argument put forward by the school of workers who consider the role of secondary organisms as insignificant in 'open' pulmonary tuberculosis is

Experiment	Aerobe	+ Anaerobe	+ T <sup>+</sup> B	Termination	Lesions at autopsy	Initial weight in grammes	Final weight in grammes
I (i) (Aerobe ± T <sup>+</sup> B)	Streptococcus, 0.5 cc	Nil	4S-0.01 mg	Death in 25 days	Inguinal glands enlarged and caseous Smears show T <sup>+</sup> B <sup>++</sup> Spleen and other organs nil Haemoculture negative	325	247
(ii) Do	Do	"	"	" " 23 "	Do	280	215
II (i) (Anaerobe + T <sup>+</sup> B)	Nil	Cst-0.5 cc	"	" " 20 "	Do	275	220
(ii) Do	Nil	"	"	" " 22 "	Do	280	230
III (i) (Aerobe + Anaerobe + T <sup>+</sup> B)	Streptococcus, 0.5 cc	"	"	" " 18 "	Do	285	220
(ii) Do	Do	"	"	" " 18 "	Do	275	195
IV (i) (Polyerobe + T <sup>+</sup> B)	Do Staphylococcus aureus, 0.5 cc	Nil	"	" " 21 "	Do	278	185
(ii) Do	Do	Nil	"	" " 24 "	Do	275	230
V (i) (Polyaerobe anaerobe + T <sup>+</sup> B)	Do	Cst-0.5 cc	"	" " 21 "	Do	290	185
(ii) Do	Do	"	"	" " 21 "	Do	286	205

	Total number experiments (gunner-pigs)	TUBERCLE BACILLI FOUND IN			Average duration of life	Average loss of body weight
		Inguinal gland only	Inguinal gland and spleen	Other organs		
I Aerobes + T B	49	40	9	Nil	23 days	82.3 grammes
II Anaerobes + T B	28	23	5		23 "	80.9
III Mono-aerobe + Anaerobe + T B	38	29	9		21	76.6 "
IV Polyaerobe + Anaerobe + T B	37	28	9		23 "	81.2



that if the sputum is properly washed several times by Kitasato's method very few secondary organisms are found in smears

As a result of our experiments we are inclined to state that the secondary organisms certainly greatly diminish in number after each successive washing, so does the tubercle bacillus also, though much less quickly. In cases with nummular sputum, very often only tubercle bacilli are seen in smears. But if these sputa are cultured, more than one variety of secondary bacteria will be found on the surface of the medium. The slowness in the reduction of the number of tubercle bacilli in sputum by repeated washing may be due to the adhesive property of the fat-enveloped bacilli.

Because tubercle bacilli are washed out more slowly than other organisms in the sputum, Bezançon, Chevalley and other (1923) failed to find secondary organisms in smears (in 37 of 58 cases). They also corroborated these results by examining the contents of cavities where they found only tubercle bacillus in 10 out of 13 cases examined. They were, therefore, led to think that, in a majority of cases, secondary organisms do not play any rôle in the tubercular process. But they admitted that a real association existed between the tubercle bacillus and aspergillosis, monilia, pneumococcus and anaerobes. On the other hand, such a careful technician as Veillon, in collaboration with Repaci, found (1912) that the scrapings from cavity walls showed secondary organisms in the majority of cases. According to him, pure tubercle bacillus infection was rare. They classified the cases into 2 groups —

- (1) those in which aerobic bacteria predominated, and
- (2) those where anaerobic bacteria took a more prominent part. They are usually present in putrid processes and cause much more damage to the lung tissue than aerobic organisms which do not seem to do much damage to the tissues surrounding a cavity.

Wassermann and Cornil compared the flora in tubercular sputum with that in the contents of cavities and found a large number of organisms which were present in both the places.

Inman (1912), in his studies on the subject, considered the tubercle bacillus as the predominating agent in 'open' cases of lung tuberculosis, but he was convinced from his data regarding the determination of the opsonic index that secondary organisms played a part in all 'resting febrile' and a majority of 'ambulant febrile' cases.

The importance of the part played by secondary bacteria in pulmonary tuberculosis in certain types of cases (e.g., cases with periodical exacerbations of temperature without any apparent cause) has been emphasized by Wingfield (5) (1923), Young (6) (1924) and others.

In 1924, Courmont and his associates at Lyons, in the course of their studies, found that secondary infections were responsible for symptoms like hectic fever and aggravation of the general condition in 20 per cent of cases, while in the remaining 80 per cent they thought the tubercle bacillus alone was sufficient to

produce every form of anatomical lesion in the lungs. They found vaccine treatment to be of benefit where the lesions were not severe or extensive. Their observations were limited to aerobic organisms only.

Ulcers of the lungs and intestines can hardly avoid pollution. But the presence of one or more of the associated bacteria is not sufficient to assign to them the rôle of a pathogenic association. To avoid the saprophytic organisms, a thorough washing of the sputum is a necessity, but it is not fair to conclude that simply because other bacteria besides the tubercle bacillus, are washed out, they do not play any role in association with the tubercle bacillus in the tubercular process. By our association experiments on guinea-pigs, we have shown that the association of aerobic and of aero-anaerobic bacteria sometimes augments the lesions. We have further shown that the duration of life of the animals inoculated with the tubercle bacillus along with secondary organisms is reduced by half in most of the cases, as compared with that of the control animals inoculated with the same dose of the tubercle bacillus alone. From our experiments we were not able to observe any gross alteration of the site of inoculation leading to such event. We are, therefore, led to believe that the association of secondary bacteria *in vivo* increases the virulence of the tubercle bacillus.

#### CONCLUSIONS

1 Repeated washings of tubercular sputum diminish the number of tubercle bacilli by only 15 per cent whereas 50—60 per cent of secondary organisms are washed out by 4 successive washings, 60—80 per cent by 5 washings and 80—90 per cent by 8—10 washings in physiological saline.

2 Even if secondary organisms are rare in smears after being washed, cultures yield a fair number of organisms.

3 Secondary organisms seem to be more frequently present in cavity cases in this country than in Europe.

4 The cellular elements in sputum are much reduced in the process of washing, but the relative proportion of leucocytes in differential count remains unaltered.

5 The differential count of leucocytes in the sputum of open pulmonary tuberculosis does not give any reliable indication as to the severity or nature of a case.

6 Of secondary aerobic bacteria, *Streptococcus hemolyticus* is as common as *Streptococcus non-hemolyticus*. No difference between the two in virulence could be ascertained by animal inoculations either with other aerobic or anaerobic bacteria or with the tubercle bacillus. The associations have been usually polymicrobial.

7 Anaerobic associations are fairly common (11 out of 40 cases) in tubercular sputum in this country, but being of mild pathogenicity, they do not seem to take any active part in the anatomo-pathological process, except in gangrenous or putrid cases. The different varieties found have been described.

8 The association of aerobic with anaerobic bacteria isolated from the sputum aids each other's action and augments the lesions in inoculated guinea-pigs in a fair number of cases

9 The association of secondary organisms seems to heighten the virulence of the tubercle bacillus in inoculated guinea-pigs. It is quite possible that the same phenomenon takes place in tubercular cavities in the human body, apart from their action in hastening the ulcerative processes

10 Attention is drawn to the results of our epidemiological studies where imperfect immunization of the population in India has been put forward as a factor in producing the anatomo-pathological picture in various tubercular lesions

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PLATE LXI



Fig 1



Fig 2

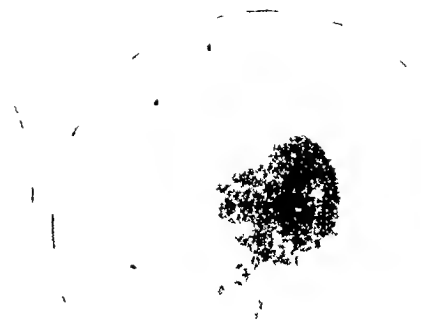


Fig 3

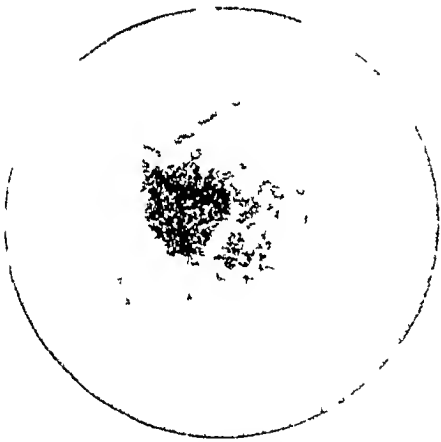


Fig 4

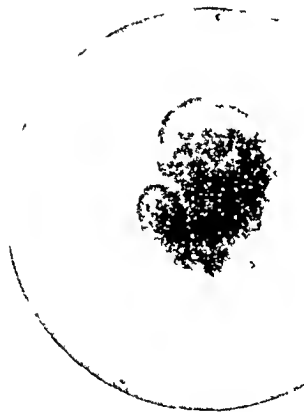


Fig 5

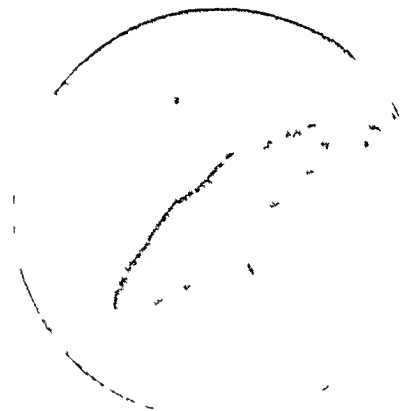


Fig 6



Fig 7



Fig 8



Fig 9

### EXPLANATION OF PLATE LXI

Appearance of Colonies of Obligatory Anaerobes in Deep Agar

- |     |   |   |
|-----|---|---|
| Fig | 1 | Cst Cocci in short chains and clumps ( $\times 12.5$ )        |
| „   | 2 | 16P Cocci in clumps ( $\times 12.5$ )                         |
| „   | 3 | 18t Cocci in short chains and clumps ( $\times 12.5$ )        |
| „   | 4 | 17P Cocci in small clumps ( $\times 12.5$ )                   |
| „   | 5 | 11t Cocci in clumps ( $\times 12.5$ )                         |
| „   | 6 | 23t Cocci in short chains and as diplococci ( $\times 12.5$ ) |
| „   | 7 | 20t Cocci in short chains and diplococci ( $\times 12.5$ )    |
| „   | 8 | 15t Cocci in short chains and clumps ( $\times 12.5$ )        |
| „   | 9 | 8t Cocci in short chains and clumps ( $\times 12.5$ )         |

#### EXPLANATION OF PLATE LXII

- Fig 1 11L Appearance of Gram-stained cocci  $\times 450$  \*  
„ 2 15t Appearance of Gram-stained cocci  $\times 450$   
„ 3 20t Appearance of Gram-stained cocci  $\times 450$
- 

\*The appearance of the microbes became hazy under the photomicrographic apparatus by increasing the magnification. We were, therefore, obliged to take a photograph under a lower power.







# ON THE DESICCATION OF ANTIVENOMOUS SERUM AND THE VALUE OF THE DRIED PRODUCT AS AN ANTIDOTE AGAINST SNAKE BITE

## Part I

BY

SURINDAR NAND LAL, I M D

(*From the Central Research Institute, Kasauli*)

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## INTRODUCTION

ANTIVENOMOUS Serum, as prepared at the Central Research Institute, consists of the untreated serum, issued in its original form, of horses highly immunized against the mixed venoms of Cobra and Russell's Viper. This serum shares with all other sera of this nature the defect that after some years its potency gradually diminishes, while with ageing a somewhat heavy deposit of the serum proteins is formed, so that the antivenin, even though it may still retain its original potency to a considerable degree, is unsuitable, without previous filtration, for intravenous injection by which route its curative properties are best manifested.

I have been able to find little published work on the use of desiccated antitoxic sera as therapeutic agents or with reference to the retention of their potency over long periods of time, but that even so unstable a substance as complement suffers relatively little loss, and retains its titre unimpaired for long periods in dried serum is evident from the work of Koenigsfelds and other investigators, especially in Germany. There seems no reason, therefore, why the therapeutic employment of desiccated antivenomous serum should not be practicable.

With regard to this serum, it has, in fact, long been the practice of the Pasteur Institute of Lille to issue antivenomous serum in the dry state. This must have been instituted somewhere between the years 1901 and 1907, in a small brochure published at Lille in the former year and entitled 'Notice sur le

Serum Antivénimeux et sur le Traitment des Morsures de Sèrpents' it is mentioned (pp 18-19) that the serum is issued in the usual liquid form, Calmette, however, in his book 'Les Vénins, les Animaux Vénimeux et la Sérothérapie Antivénimeuse,' published in 1907, mentions on page 260 that dried antivenomous serum is issued from the Pasteur Institute. According to Calmette, the dried serum remains unaltered almost indefinitely. He mentions a 10 per cent solution as suitable for injection. He does not, however, refer to the method of drying the serum nor to any comparative potency tests of the original and the dried serum which may have been carried out, nor have I been able to find any reference to such work in the literature.

More recently, some unpublished experiments, carried out by Caus and Iyengar in India during their work on the concentration of antivenin, included a series of experiments with desiccated serum. Father J. F. Caus, S. J., has very kindly placed his notes at my disposal and the foregoing remarks are extracted from these notes. The problem they were engaged on was the possibility of obtaining a definite concentration of the antivenin contained in the serum of immunized horses by a variety of methods, including desiccation either of untreated serum or of serum previously treated in various ways. From the results of the few experiments they carried out on the lines that I have followed in this present work, it appeared that desiccation *in vacuo* over sulphuric acid was readily brought about and that the serum when re-dissolved in approximately its original quantity of fluid more or less retained its original potency, but that no useful degree of concentration could be obtained by this means, this line of inquiry was therefore not followed up by them and comparative tests to estimate the relative keeping properties of the original and the desiccated serum were not done. In their work Caus and Iyengar re-dissolved the dried product in 0.4 per cent saline, and although solution occurred readily there was in all cases a considerable precipitate which was apparently protein thrown out of solution by the excess salt.

The object of the work I have undertaken is first the determination of the most suitable and practical method of desiccating antivenomous serum in such a way that the dried product loses little or none of its potency in the process of drying, while it is still capable of being readily re-dissolved in a solution suitable for intravenous injection, secondly, evaluation of the respective keeping property of the two sera by means of repeated potency tests at intervals of time, with the dried product and its corresponding untreated serum.

#### METHOD OF PREPARING DRIED ANTIVENIN

The serum used throughout these experiments was obtained in the manner which is in routine use at the Central Research Institute, Kasauli, for the preparation of antivenin. The immunized horse, after estimating roughly the titre of the serum, is bled aseptically into sterile conical flasks of about two litres capacity. The serum is allowed to separate out of the clot and is decanted over

into a similar flask whence it is filled into 40 c c capsules by Maynard's method. The serum of several horses was collected in this way, some being that from one horse only and some the pooled serum of 3 or 4 horses. Some of the serum collected was set aside for the purpose of comparative potency tests during the course of the experiments while the remainder was used for the preparation of the dried product. Each batch of serum before being used for the experiments was tested for sterility, and its potency against cobra venom was estimated by a biological test in pigeons.

Various methods of drying the serum were tried before one which appeared to answer all requirements was devised. Of the methods for drying the serum, the most obvious seemed to be the application of heat. A quantity of liquid serum was placed in sterile Petri dishes and kept till dry at temperatures between 37°C and 42°C and also at room temperature. Attempts to dry the serum in this way were, however, soon abandoned, for at room temperature the time required was very great and contaminations were numerous, while at the higher temperatures, although desiccation occurred fairly rapidly and without many contaminations the resultant product dissolved either very slowly, or solution was incomplete, while such solutions as were obtained were far from clear.

The method finally decided upon, and which gave the most successful results, is the following —

The apparatus consisted of two glass-dishes, one an ordinary 1 inch deep by 4½ inches in diameter Petri dish and the other a deeper and slightly larger dish 2 inches deep by 5 inches in diameter. Two flattened glass rods are fixed with plasticine over the mouth of the larger dish forming a stand on which the smaller Petri dish rests. A bell-jar with two openings and a ground-glass plate complete the apparatus required.

A quantity (about 40 c c) of the serum, previously tested for sterility, is carefully measured with a sterile pipette and placed in the sterilized Petri dish and covered with the lid. The dish containing the serum is then placed over the larger dish in which is put about 200 c c of concentrated sulphuric acid. The whole is then put on the ground-glass plate and covered with the bell-jar which is firmly luted down with grease. One opening of the bell-jar is fitted with a glass stopper and stop-cock, the other opening has a rubber cork through which passes a glass tube connected to a short length of rubber tubing plugged at its other end with a glass rod. The bell-jar is then connected to a Geryk pump and exhausted until the rubber tube is completely flattened. This tube then acts as a test for the holding of the vacuum. The whole is left at room temperature in Kasauli from 15°C to 22°C until dry. If the rubber tube indicated loss of vacuum, the jar is again exhausted and the sealing looked to. From time to time the sulphuric acid in the lower dish is gently agitated to mix its contents.

When completely dried, the serum residue is scraped off with a sterile knife, carefully weighed and stored in a sealed tube. The dried product has the usual crystalline appearance of dried serum.

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When treated as above, desiccation of 40 c c of serum is complete in some 43 hours (average of 8 experiments), solution is readily effected in distilled water and the resulting solution is free from solid particles and shows only a slight opalescence, and is therefore quite suitable for intravenous injection

Apparatus for Desiccating Antivenomous Serum



- A Small Petri dish containing antivenomous serum
- B Large Petri dish containing sulphuric acid
- C Bell-jar
- D Glass stopper with stop-cock
- E Rubber tubing used as an indicator for holding vacuum in the jar
- F Ground glass slab

Table I shows the time required for the complete desiccation by the above method of 40 c c of serum

TABLE I

Serial number	Quantity of serum in c.c	Hours
1	40	44
2	40	42
3	40	43
4	40	42
5	40	43
6	40	44
7	37	43
8	44	44

Average time for 40 c.c. = 43.1 hours

The percentage of solids obtained from the serum on desiccation was about the same as found by Burrows and Cohn. It was found, however, that this percentage was slightly higher when the pooled serum of several horses was used than in the case of the serum from a single horse. Table II shows the percentage of solids obtained in each case.

TABLE II

(A) Mixed serum from 4 horses

Liquid serum c.c	Solids grammes	Solids per cent
40	4.19	10.475
40	4.21	10.525
40	4.11	10.275
Average solids per cent		10.425

(B) Serum from 1 horse only

Liquid serum c.c	Solids grammes	Solids per cent
40	3.70	9.250
40	3.770	9.425
39	3.665	9.372
37	3.495	9.445
44	4.200	9.545
Average solids per cent		9.407

The figures in Table II (B) agree very closely with those obtained by Caus and given in unpublished notes referred to above.

The dried product is readily soluble in distilled water or saline solution. For the purpose of these experiments, distilled water was used so as to avoid altering the salt content of the original serum. Solution is effected by gentle shaking.

As would be expected, the time taken for complete solution of the dried product varies according to the final concentration required. A certain amount of concentration of the serum can apparently be effected by dissolving the dried serum in less fluid than its original content, but the higher concentrations are too thick for injection, and the effect of such concentration as can be obtained on the potency of the serum has not yet been tested.

The time required for solution is as follows —

- (a) Made up to original quantity of serum, i.e., about 1 in 10, 5 minutes
- (b) Dissolved to make a 1 in 5 solution, 8 minutes
- (c) Dissolved to make a 1 in 4 solution, 15 minutes

The dried serum will also dissolve in 1 in 3 solution, but this and the 1 in 4 solution are too viscous for injection. When solution was effected in sterile distilled water, the pH of the re-dissolved product was about 8.0, the original serum being pH 7.7.

#### EXPERIMENTAL WORK

For all the experiments described below, which are to be regarded as of the nature of preliminary tests, the serum was dried as described above and stored in sealed test tubes at room temperature (in Kasauli about 25° to 96°F). In every case, the serum after being drawn and bottled, was tested aerobically and anaerobically for sterility. When required for the tests, the dried product was re-dissolved in sterile distilled water in order to avoid adding an excess of salt. The solution was made up to the original quantity of the liquid serum from which the dry sample was obtained. The re-dissolved serum was tested aerobically and anaerobically for sterility.

Throughout the work now to be described both the original antivenin and the dried serum were obtained from the same bleeding of the same horse and the results are therefore comparable.

The potency tests described here were all carried out on pigeons according to the routine test employed at the Central Research Institute for estimating the potency of antivenin. In this test 1 c.c. of the serum to be tested is mixed in a test tube with 1 c.c. of venom solution dissolved in normal saline in different concentrations. The mixtures are incubated for 30 minutes at 37°C and the whole (2 c.c.) injected into the breast muscles of a series of pigeons of 300 grms weight. The tests are carried out against cobra venom only. With each test a control is put up for the minimum lethal dose of the sample of venom used.

The readings are taken after 24 hours, the difference between the M. L. D. of the venom and the maximum test dose survived by the pigeons is regarded as the amount of cobra venom neutralized by 1 c.c. of the serum.

The following tables show the potency of the original and dried serum at different periods —

TABLE III

*Potency against cobra venom of (a) freshly prepared antivenum, and (b) freshly prepared dried antivenum*

Date of test	Pigeon number	Test dose cobra venom	Result	REMARKS		
(a) Freshly prepared antivenum						
13-2-27	1	0.7 mg	Lived	The solution of the dried antivenum used for this test was made on 7-2-27		
	2	0.8 "			"	
	3	0.9 "			"	
	4	1.0 "			"	
	5	1.1 "	Died			
	6	1.2 "	"			
(b) Freshly prepared dried antivenum						
13-2-27	7	0.7 mg	Lived		The solution of the dried antivenum used for this test was made on 7-2-27	
	8	0.8 "				"
	9	0.9 "				"
	10	1.0 "				"
	11	1.1 "	Died			
	12	1.2 "	"			
Controls						
13-2-27	13	0.2 mg	Died	The solution of the dried antivenum used for this test was made on 7-2-27		
	14	0.3 "				"
	15	0.4 "				"
	16	0.5 "				"

The minimum lethal dose of the venom = 0.2 mg

1 cc freshly prepared antivenum neutralizes 0.8 mg cobra venom

1 cc freshly prepared dried antivenum neutralizes 0.8 mg cobra venom



TABLE IV

Potency against cobra venom of (a) antivenum after storage for 3 months, and  
(b) dried antivenum after storage for 3 months

Date of test	Pigeon number	Test dose cobra venom	Result	REMARKS	
	(a) <i>Antivenum 3 months old</i>				
13-5-27	17	0.7 mg	Lived	The solution of the dried antivenum used for this test was made on 9-5-27	
	18	0.8 "			
	19	0.9 "			
	20	1.0 "			
	21	1.1 "			
	22	1.2 "			
		Plus 1 c c serum			
	(b) <i>Dried antivenum 3 months old</i>				
13-5-27	23	0.7 mg	Lived		
	24	0.8 "	"		
	25	0.9 "	"		
	26	1.0 "	"		
	27	1.1 "	"		
	28	1.2 "	Died		
		Plus 1 c c serum			
	<i>Controls</i>				
13-5-27	29	0.1 mg	Lived		
	30	0.2 "	Died		
	31	0.3 "	Cobra venom	"	
	32	0.4 "		"	
	33	0.5 "		"	

The minimum lethal dose of the venom was 0.2 mg

1 c.c. of 3 months old antivenum neutralized at least 1.0 mg cobra venom

1 c.c. of 3 months old dried antivenum neutralized 0.9 mg of cobra venom

TABLE V

Potency against cobra venom of (a) antivenin stored for 1 year, and (b) dried antivenin stored for 1 year

Date of test.	Pigeon number	Test dose cobra venom	Result	REMARKS	
	(a) <i>Antivenin 1 year old</i>				
13-2-28	34	12 mg	Lived	The solution of the dried antivenin used for this test was made on 12-1-28	
	35	13 "	"		
	36	14 "	"		
	37	15 "	Died		
	38	16 "	"		
	39	17 "	"		
	40	18 "	"		
	41	19 "	"		
	42	20 "	"		
	(b) <i>Dried antivenin 1 year old</i>				
13-2-28	43	12 mg	Lived		
	44	13 "	"		
	45	14 "	"		
	46	15 "	Died		
	47	16 "	"		
	48	17 "	"		
	49	18 "	"		
	50	19 "	"		
	51	20 "	"		
	<i>Controls</i>				
13-2-28	52	0.1 mg	Lived		
	53	0.2 "	"		
	54	0.3 "	"		
	55	0.4 "	Died		
	56	0.5 "	"		

The minimum lethal dose of the venom was 0.4 mg

1 cc. of 1 year old antivenin neutralized 10 mg of cobra venom

1 cc of 1 year old dried antivenin neutralized 10 mg of cobra venom

TABLE VI

*Potency against cobra venom of (a) antivenum stored for 2 years, and (b) dried antivenum stored for 2 years*

Date of test	Pigeon number	Test dose cobra venom		Result	REMARKS	
15-2-27	(a) <i>Antivenin 2 years old</i>					
	57	10 mg	}	Lived	The solution of the dried antivenin used for this test was made on 13-2-29	
	58	1·1 "		Died		
	59	1·2 "		"		
	60	1·3 "		Very ill but alive		
	61	1·4 "				Died
	62	1·5 "				"
15-2-29	(b) <i>Dried antivenin 2 years old</i>					
	63	10 mg	} Plus 1 c c serum	Lived		
	64	1·1 "		Died		
	65	1·2 "		"		
	66	1·3 "		"		
	67	1·4 "		"		
	68	1·5 "		"		
<i>Controls</i>						
	69	0·2 mg	} Cobra venom	Lived		
	70	0·3 "		Died		
	71	0·4 "		"		
	72	0·5 "		"		

The minimum lethal dose of the venom was 0·3 mg

1 cc of 2 years old antivenum neutralized probably 0·7 mg cobra venom The survival of No 60 pigeon was probably accidental

1 cc of 2 years old dried antivenum neutralized 0·7 mg cobra venom

TABLE VII  
Repeat test with 2 years old sera

Date of test	Pigeon number	Test dose cobra venom	Result	REMARKS
(a) <i>Intervenin 2 years old</i>				
19-2-29	73	10 mg	Lived	
	74	11 "	"	
	75	12 "	"	
	76	13 "	Died	
	77	14 "	"	
	78	15 "	"	
(b) <i>Dried antivenin 2 years old</i>				
19-2-29	79	10 mg	Lived	The solution of the dried antivenin used for this test was made on 13-2-29
	80	11 "	"	
	81	12 "	Died	
	82	13 "	"	
	84	14 "	"	
	85	15 "	"	
<i>Controls</i>				
19-2-29	86	0.2 mg	Lived	
	87	0.3 "	Died	
	88	0.4 "	"	
	89	0.5 "	"	

The minimum lethal dose of the venom was 0.3 mg

1 cc of 2 years old antivenin neutralized 0.9 mg cobra venom

1 cc. of 2 years dried antivenin neutralized 0.8 mg cobra venom

TABLE VIII

*Repeat test with 2 years old sera*

Date of test	Pigeon number	Test dose cobra venom		Result	REMARKS		
28-2-29	(a) <i>Antivenum 2 years old</i>						
	90	1.0 mg	} Plus 1 c c serum	Lived			
	91	1.1 "		"			
	92	1.2 "		Died			
	93	1.3 "		"			
	94	1.4 "		"			
	28-2-29	(b) <i>Dried antivenum 2 years old</i>				The solution of the dried antivenum used for this test was made on 13-2-29	
		95	1.0 mg	} Plus 1 c c serum			Died
		96	1.1 "				"
		97	1.2 "				"
98		1.3 "	"				
99		1.4 "	"				
100		1.5 "	"				
28-2-29	<i>Controls</i>						
	101	0.2 mg	} Cobra venom	Lived			
	102	0.3 "		"			
	103	0.4 "		Died			
	104	0.5 "		"			

The minimum lethal dose of the venom was 0.4 mg

1 c c of 2 years old antivenum neutralized 0.7 mg cobra venom

1 c c. of 2 years old dried antivenum neutralized less than 0.6 mg cobra venom

## SUMMARY

When the above results are considered, it must be admitted that they are somewhat disappointing. A considerable degree of irregularity in the readings, even more than is usually met with in such tests, is apparent and has marred the results, while although there has been exhibited no great difference in the neutralizing power of the untreated and the dried serum respectively, there is no evidence as yet that the dried product retains its potency any better than the original serum, since on no occasion did the dried serum show a higher neutralizing power than the original untreated serum of the same age.

It has been shown, however, by Anderson and Caus that the potency of ordinary antivenomous serum is fully retained or even increased up to a period of at least two years, so that it is evident that a further series of tests after considerably longer periods will be required before any appreciable difference in the potency of the two sera is likely to be met with as the result of storage only.

At the same time it appears from the results obtained immediately after desiccation (Table III) that no loss of potency is incurred in the process of drying, and further that there is no loss of potency up to a period of one year (Table V). By this time (one year) ordinary serum contains a very considerable deposit and is unfit for intravenous injection without previous filtration, while the solution of dried serum is practically clear so that in this case the dried product has a distinct advantage.

Certain of the results (Tables VII and VIII) appear to indicate that after being in solution a certain time, the dried product begins to lose potency, but this point requires further investigation.

## CONCLUSIONS

(1) Antivenomous serum can be rapidly desiccated *in vacua* over sulphuric acid and no appreciable loss of potency is incurred in the process of drying.

(2) Solution of the dried product is readily effected in distilled water and the resultant solution is practically clear. The clearness of the solution is not affected by storage of the dried serum, at least up to two years.

(3) No loss of potency in the dried serum was found to have occurred after storing for one year, by which time the original serum, though equally potent, contained a considerable deposit and was no longer suitable for intravenous injection.

(4) No evidence has as yet been obtained that the dried product retains its potency any better than the original serum.

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\* Seen in abstracts only

# OSTEOMALACIA (LATE RICKETS) STUDIES

## Part II

### THE BLOOD PICTURE

BY

DAGMAR CURJFL WILSON, M D (Glas ), D P H (Camb ), W M S (Retd ),  
*In charge Osteomalacia Inquiry, Indian Research Fund Association,*

AND

GULBAI P PATEL M B, B S (Boin & Lond ), L M (Dub ),  
D T M & H (Cal ), W M S

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*Conditions of the research*—As explained in a previous paper(1) the object of this research is to study cases of osteomalacia in the early stages of the disease, while still amenable to treatment. In the same communication it was shown that in India, as in other parts of the world, there is no hard and fast line of demarcation between so-called 'late' rickets the disease setting in from the tenth year, and osteomalacia. X-ray findings show that the differences noted clinically depend rather upon the age of onset than upon any essential difference in the morbid processes concerned, and justify the grouping of certain signs and symptoms as diagnostic of various stages of osteomalacia (late rickets). The close relation of early rickets the disease of the first and second years of life, to late rickets was also recognized, and intermediary cases noted.

In connection with the osteomalacia inquiry, investigations are being carried out at various centres in the Punjab and in Delhi Province. The series of blood examinations, together with stool and urine findings recorded in this present paper, were carried out on patients attending the Osteomalacia Clinic at Simla by one of the authors, (G P P), whose services were very kindly lent for this purpose by the Women's Medical Service, India.

*Conditions of examination*—A series of forty-six cases of osteomalacia (late rickets) controlled by X-ray examinations were studied at the Simla Osteomalacia Clinic, the blood in every case being examined on the patient's admission to the



clinic and in a third of the cases again after varying periods, to note the effects of treatment

*Age and sex distribution*—All the patients were female, women and girls whose ages ranged from ten to forty years. In considering the differential blood count, since a relative lymphocytosis is said to occur during every menstrual period, in no case was the blood examined while the patient was menstruating. In addition four cases of rickets occurring under ten years of age were also examined.

*Caste*—Twenty-five patients were Mohammedan and twenty-four Hindu (included in the latter group are three of the low Chamar caste), one patient was a Christian from Goa.

*Marriage*—Forty-two were married women and girls, thirty-three of whom had undergone at least one pregnancy, four had no children, in addition five of the younger married girls were still living in their mother's house.

*Altitude*—The patients included women who had lived all their lives at high altitudes, as well as Indian women and girls of other castes who were staying in the hills for varying periods. Since at the time of examination all the patients were living in the neighbourhood of Simla at a height of 7,000 to 7,500 feet, the effect of the altitude on the blood picture had to be considered. The results were therefore controlled by examination of normal cases from similar classes of women. The normal blood count in Simla was thus found to be—

Average of 10 cases	Polymorphonuclear 56.5 per cent
Red blood cells—5,710,000	Metamyelocytes 3 per cent
White blood cells—6,620	Eosinophiles 4 per cent
Hæmoglobin (Tallquist) 79 per cent	Basophiles nil
Differential count Using Leishman's stain	Lymphocytes 31.5 per cent
	Large mononuclear 5 per cent

The normal Western blood picture is given (2) as

Red blood cells	
Men	5,000,000
Women	4,800,000
White blood cells	
Men	7,500
Women	8,000
Hæmoglobin (Gower's)	82 per cent

Schilling (3) gives the following as normal figures for the leucocytic blood picture

Leucocytes 6,000 to 8,000 per cmm  
Neutrophile granular cells 67 per cent

[Composed of 63 per cent polymorphonuclears and 4 per cent of old metamyelocytes ('band' forms), young metamyelocytes being absent]

Eosinophiles 3 per cent  
Basophiles 1 per cent

Lymphocytes 23 per cent

Monocytes 6 per cent

As the result of considerable experience of blood examinations among women in North India it has been noted by one of the authors (G P P) that in normal cases the average total white cell counts tends to be slightly lower than among women of Western races.

The altitude therefore in the cases examined at Simla may be said definitely to lead to a higher hæmoglobin percentage and red cell count, and to raise to a slight extent the white cell count. Therefore in considering the blood picture in this series of cases of osteomalacia (late rickets) the average augmented readings found at Simla have been taken as the normal for comparison.

*Results*—In the series of forty-six cases of osteomalacia (late rickets), the following results of examination were observed—

1 A slight degree (about 70 per cent) of anæmia was usually present, later in cases re-examined where clinically improvement was evidenced the hæmoglobin was found to have increased to about 85 per cent.

2 There was a slight increase in the total number of white cells, except in cases complicated by pregnancy after the fifth month where, as might be expected, the increase was more marked.

3 Some cases showed a fairly marked lymphocytosis (35 to 50 per cent) with no apparent relation clinically to the acuteness of the case. At subsequent examination this lymphocytosis appeared to have been a transitory phase in those cases showing marked clinical improvement (lessening of pain, increased freedom of movement etc.), since the next blood picture showed a decrease in the lymphocytes (33 to 40 per cent) and an increase (40 to 55 per cent) in the polymorphonuclears. In cases where there was no evidence of this lymphatic reaction, no clinical improvement was evident during the refractory period. It was noted in some cases complicated by gonorrhœal infection that the patients' reaction to treatment was very slow.

4 An increase (in some cases up to 10 per cent) of metamyelocytes and myelocytes was noticed in nineteen cases, with no apparent relation clinically to the acuteness of the symptoms.

5 The occasional appearance (20 per cent of cases) in the blood of a few endothelial cells was noted.

6 The blood picture in the four cases of rickets under ten years of age came into line with that of some of the older patients showing lymphocytosis.

*Examination for concurrent infections*—In order to eliminate other factors which might affect the blood picture, microscopical examination of the stools and bacteriological examination of the urine was also undertaken.

*Stool examinations*—Seventeen cases were examined for protozoa and helminths with the following results—

2 cases showed a scanty infection of *A. lumbricoides*

11 cases varying degrees of encysted *E. histolytica*

1 case a scanty infection of *Lambia intestinalis*

13 cases varying amounts of *Blastocystis hominis* and *Sarcinae*, all much in excess of the normal

*Urine examinations*—Seventeen cases were examined by culture of catheter specimens of urine

1 case was sterile

15 cases showed yeast and staphylococci

3 cases *B coli* strains, 1 being *B coli communis* and 2, *B acid lactici* (Huppe) (4)

1 case non-hæmolytic streptococci, a luxuriant growth

1 case the same but extremely scanty

The staphylococci and yeasts found in all except one of the catheter specimens may perhaps be due to the difficulty of sterilizing the entrance of the female urethra

### DISCUSSION

The position with regard to changes in the circulating blood in deficiency (avitaminotic) disease is still not fully worked out

In food deficiency of which rickets is one of the most obvious signs (5) a lymphocytosis is described, as also in scurvy, (6) but Piney (7) states that we are still quite ignorant of the relationship of the anæmia which is present to the rickets

In beri-beri (8) there is always some anæmia which may become severe, there is little change in the number of leucocytes, but slight transient lymphocytosis is common

In pellagra which is by some regarded as a deficiency disease, (9) there is a secondary anæmia and lymphocytosis

The lymphocytosis occurring among certain patients in this series of cases of osteomalacia (late rickets) which on improvement was replaced by a polymorphonuclear increase, may be a sign of some vitaminic deficiency, but since lymphocytosis also occurs during bacterial infections (such as tuberculosis, whooping cough and typhoid fever) there is also the possibility of some infective process at work, either as a primary or secondary factor in the avitaminatic condition. The stool examinations showing excess of *sarcinae* and yeasts, may possibly indicate a faulty carbohydrate metabolism with stasis, as the majority (38 out of 50) of cases under examination were markedly constipated

The remarkable freedom from helminthic infection is probably a result of a purer water supply as compared with the plains, also many of the cases examined were of some social status living under fair sanitary conditions

As regards the urine, the two cases in which *B acid lactici* strains were isolated, if considered in conjunction with the increased *sarcinae* and yeasts of the stools, may indicate a fermentative process going on in body. In this connection the presence of occasional endothelial cells in the peripheral blood in certain cases, if derived from damaged capillaries, may explain leakage of organisms from the gut

## CONCLUSION

1 A study of the blood picture in a series of forty-six cases of osteomalacia (late rickets) showed that in patients where clinical improvement was evident, the increased lymphocytes found in the differential count at the first examination were replaced at subsequent examination after about three weeks' treatment by a varying but definite increase of polymorphonuclear leucocytosis. Cases where during the period under observation no clinical improvement was noted, did not show this reaction.

Together with the clinical improvement there was a definite increase in the haemoglobin percentage. The relation of these changes to those found in some deficiency diseases is discussed.

2 Coincident examination of the stools and urine gave evidences of fermentative processes going on in the body, suggesting the possibility of an infective process in the intestine either as a primary factor or dependent on an avitaminotic condition.

## CASES

Examples of cases of osteomalacia (late rickets) showing changes in the blood picture lymphocytosis giving place to leucocytosis associated with clinical improvement of symptoms.

*Serial No 4*—Molani, et 25 Hindu Chamar age at marriage 17, age at onset of disease 21 Non-purdah 4-part Pregnant 6 months Social conditions very poor

	10-5-29	7-6-29
Hæmoglobin	70 per cent	70 per cent
Red blood cells	3 650 000	
White blood cells	5,600	9,000
Differential count—		
Polymorphonuclear	62 per cent	74 per cent
Large mononuclear	2 "	2 "
Lymphocytes	36 "	24 "
Macrocytes and microcytes present	A few macrocytes	and slight
Poikilocytosis and vacuolated red cells noted	basophilia present	

*Serial No 10*—Muniran, et 19, Hindu Chamar, unmarried age at onset of disease 17, non-purdah, social conditions poor

	13-5-29	10-6-29
Hæmoglobin	60 per cent	80 per cent
Red blood cells	2 740 000	
White blood cells	7,500	7,400
Differential count—		
Polymorphonuclear	38 per cent	52 per cent
Large mononuclear	4 "	7 "
Eosinophiles	6 "	6 "
Lymphocytes	52 "	30 "
Metamyelocytes		5 "

*Serial No 19*—Khatun Anwar æt 30, Punjabi, Mahommedan age at marriage 19, age at onset of disease 27, observes purdah, 3-para Social conditions favourable

	17-5-29	6-6-29
Hæmoglobin	75 per cent	85 per cent
Red blood cells	5 050,000	
White blood cells	7,000	6,000
Differential count—		
Polymorphonuclear	43 per cent	55 per cent
Large mononuclear	4 "	2 "
Eosinophiles	2 "	1 "
Lymphocytes	51 "	40 "
Metamyelocytes		2 "

Examples of two acute cases which failed to show any lymphocytosis and where after treatment, at subsequent examination there was as yet no marked difference in the blood picture, thus showing that there is not in every case, even during an acute phase, a typical blood picture of osteomalacia (late rickets)

*Serial No 12*—Laxmi æt 18, Hindu Chamar, unmarried, age at onset of disease 11, non-purdah, social conditions very poor Typical example of the disease starting in the late rickets age period, 3 other members of her family were suffering from a similar condition

	4-5-29	7-6-29
Hæmoglobin	75 per cent	75 per cent
Red blood cells	4,730 000	
White blood cells	10,400	5,000
Differential count—		
Polymorphonuclear	60 per cent	64 per cent
Large mononuclear	6 "	
Eosinophiles	2 "	2 "
Lymphocytes	30 "	34 "
Myelocytes	2 "	

Vacuolated red blood cells present

*Serial No 15*—Munsa, æt 19 Hindu, zemindari from Kangra Valley, age at marriage 7, age at onset of disease 17, non-purdah 3-para, social conditions fair, typical acute osteomalacia

	14-5-29	10-6-29
Hæmoglobin	70 per cent	70 per cent
Red blood cells	7,440,000	
White blood cells	6 000	6 000
Differential count—		
Polymorphonuclear	55 per cent	55 per cent
Large mononuclear	4 "	4 "
Eosinophiles	6 "	5 "
Lymphocytes	33 "	30 "
Metamyelocytes	{ 2 old 1 young 1 "	{ 6 old 4 young 2 "

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# OSTEOMALACIA (LATE RICKETS) STUDIES

## Part III

### DIETARY FACTORS IN THE ÆTIOLOGY OF OSTEOMALACIA

BY

DAGMAR CURIEL WILSON, M D (Glas ), D P H (Camb ), W M S (Retd ),

*In charge Osteomalacia Inquiry Indian Research Fund Association,*

AND

ELLA SURIE, M SC (Lond ),

*Professor of Physiology Lady Hardinge Medical College, Delhi, working under  
the Indian Research Fund Association*

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#### INTRODUCTION AND HISTORY

THE ætiology of osteomalacia is a subject which has roused considerable interest during the last two decades. A survey of the recent work in China and India where this disease is in some areas endemic [Maxwell and Miles(1, 2), Miles and Feng(3), Scott(4a, 4b), Hutcheson and Stapleton(5, 6), Megaw(7) Green Armytage(7)] and the previous papers in the present series(9, 10), together with the post-war findings in Europe usually described as hunger osteomalacia(11) [Dalyell and Chuck(12), Bittorf(13), Looser(14), Alwens(15), Heyer(16), Burchardt-Socin(17)] suggests that osteomalacia is a true deficiency disease, and as such is but an adult manifestation of the early rickets of the first and second years of life and the late rickets starting at about the tenth year. The clinical signs of late rickets and osteomalacia in relation to bone changes as shown by X-ray examination has already been described in these series of studies, and the loss in muscular tone noted. Maxwell and Miles(2) showed that in some cases the muscular symptoms predominate and bone changes are but very slight, electrical reactions being in all cases normal. This has been experimentally shown by one of us (E S) in the case of rachitic puppies, but in this instance severe muscular atrophy also occurred in several cases.



The immediate post-war period in Europe revealed, particularly in Germany and Austria a number of cases which can now be definitely classified as hunger osteomalacia. The malnutrition was not only qualitative, but quantitative as well. Alvens(15) described cases of women in Munich in 1919 who presented clinical symptoms comparable to those of true osteomalacia except that he failed to find pelvic changes, Bittoif(13) mentions a number of cases of late rickets in boys and Heyer(16) cases of osteomalacia in both men and women which occurred in Munich. The symptoms disappeared with improved diet and administration of cod-liver oil. Heyer(16) and Loose(14) in their articles suggest that the conditions described are closely related to those of late rickets, osteoporosis, and osteomalacia, Heyer goes so far as to foreshadow the present conception of the aetiology of this disease and considers that not only did his cases present almost complete lack of calcium and phosphorus, but that some other dietetic factor or factors were involved. In Austria the work of Chick and her co-workers on rickets and hunger osteomalacia(12) is too well known to be described here, but it may be mentioned, that their independent observations endorsed the view of German investigators that hunger osteomalacia was due to a dietary deficiency and that it exhibited the same seasonal variations as rickets.

Thus the conclusions of these observers go to prove that when certain vitamins are deficient, either through dietary deficiency or lack of sunlight or both, rickets and osteomalacia are liable to manifest themselves, in spite of geographical or social differences.

The literature on late rickets and osteomalacia contains but scant evidence of families or inmates of the same house suffering from this disease, and thus some observers have been led to doubt the importance of the dietetic factor in the aetiology, in spite of the fact that Wamplar(18) and Saltzmann(19) mention family histories. In connection with the present investigation evidence has been brought forward to show that families and inmates of one house do often develop this disease in varying degrees of severity.

Lack of vitamin D in the diet or lack of sunlight are the chief causative factors, but there is also a positive factor which deserves consideration, i.e., the large cereal intake of the inhabitants of the East. Mellanby(20) has shown that some anti-calcifying substance, which he has designated as 'Toxamin' is present in many cereals. The amount of 'Toxamin' varies in different cereals and bears no relation to their calcium-phosphorus ratio. Of those tested, oatmeal has been proved to have the greatest anti-calcifying action, both experimentally on puppies, and clinically in children, particularly with regard to the incidence of dental caries\* in the latter group [May Mellanby, Pattison, Proud(21), (22), (23)]. The work of the Sheffield School has shown that the 'Toxamin' can be

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\* In textbooks it is sometimes stated though erroneously, that dental caries is rare among Indian races. As an opportunity of assessing the relationship between rickets and dental caries in school children has arisen in the course of the present investigation, the findings will be incorporated in a subsequent communication.

destroyed by certain chemical agents and neutralized by an ample sufficiency of vitamin D in the diet. It is interesting to note that Gilks and Orr(24) in studying 'The nutritional condition of the B. African native's, concluded that the physique of the tribes among which cereal formed the greater bulk of the diet, was inferior as compared with the physique of the meat eating tribes.

The endemicity of osteomalacia in certain areas in India is suggestive of dietary deficiencies as factors in its aetiology, and the purpose of the present paper is to examine the diet of those suffering from this disease in one of these areas, namely in certain districts of the Punjab.

Since lack of vitamin D in the diet, lack of sunlight or lack of both have already been shown by earlier observers to be concerned in the aetiology of osteomalacia, the diets of such patients have been examined in some detail with a view

- (1) to confirming the lack of vitamin D, either partial or absolute,
- (2) to bringing forward some evidence that an excess of cereal in the diet results in an exacerbation of the disease unless neutralized by adequate amounts of vitamin D.

#### LINES OF INVESTIGATION

The 265 cases on which this report is based are drawn from four different areas in the Punjab, Lahore, Amritsar, Simla and the upper part of the Kangra Valley, where many cases of osteomalacia are found.

The routine information obtained from each patient was as follows —

- (1) Name, date seen, present age, age at onset of disease, age at marriage
- (2) Birth place, present dwelling and any intermediate station
- (3) Religion, caste, occupation of husband or father. This includes some estimate of the financial position and consequent ability to obtain necessary food-stuffs.
- (4) Housing conditions, amount of sun obtainable and actually obtained, if purdah is observed or not.

It is extremely difficult to obtain reliable evidence with regard to the amount of sun obtained, and in some cases where the housing conditions of the patient have been known to be bad, the considerable amount of sun said to be obtained, has been discredited.

- (5) Onset of menstruation, if married, number, dates and details of pregnancies with relationship of onset of symptoms to pregnancy or lactation.
- (6) History of late rickets or osteomalacia, date and mode of onset, relation to any other clinical condition, initial and subsequent symptoms, gait, deformities, sites of pain with degrees of severity.
- (7) General impression of physical condition.

The clinical symptoms have been classified by one of us (D. C. W.) as + + + +, + +, or + according to present degrees of severity. This classification applies more to the actual acuteness of the physiological condition of the bones

than to the degree of deformity which may be of long standing and incurable. Many cases were controlled by X-ray examination.

(8) Diet

- (a) Amount of *milk* per diem
- (b) Amount of *fresh fruits* per diem
- (c) Amount of *ata* per diem
- (d) Amount of *dal* per diem
- (e) Amount of *rice* per diem
- (f) Amount of *cooked vegetable* per diem
- (g) Amount of *meat*
- (h) Amount of *fish*
- (i) *Fat* (Ghee), (Oil)

Variation, if any, in the diet, was noted

(a) *Milk*—The amount of milk taken varies, but is seldom adequate to the needs of the patients. The question of adulteration must also be borne in mind. In nearly all cases the milk is boiled before use.

(b) *Fresh fruits*—In the upper parts of the Kangra Valley fresh fruit or fresh vegetables are unobtainable unless imported and in Simla they are expensive. In other places the patients are not accustomed to the regular use of fresh fruit or uncooked vegetables. Taking an average of the total number of cases, the amount of fruit or uncooked vegetable consumed per individual per diem is practically negligible.

(c) *Ata*—This is a very variable factor, the *ata* may be coarse or finely ground whole meal, it may be comparable to the white flour in England, but all degrees of milling intermediate between the above two types are met with. The wheat flour may be adulterated in very varying proportion with millet, dal or gram. It is therefore difficult to assess the dietetic value of *ata*, but it is usually consumed in sufficient quantities to afford sufficient vitamin A and since consumed together with dal, the cereal moiety of the diet affords a sufficiency of vitamin B.

(d) *Dal*—As a rule only small quantities of this legume is consumed per diem, the actual amount is not always exactly stated by patients—‘a handful of cooked dal’ is the usual answer obtained.

(e) *Rice*—Both polished and unpolished, much less is consumed in the Punjab than in Southern India. Most of the cereals sold in the Punjab are grown within the province.

(f) *Cooked vegetables*—These may be taken once or twice per diem. One of us (E. S.) has investigated the methods of cooking in the homes and it seems to be an invariable rule to cook the vegetables for at least two hours. This leaves little chance for any survival of vitamin C.

(g) *Meat*—When eaten is usually goat and of poor quality, and this is stewed for at least four hours.

(h) *Fish* is usually difficult to procure and is seldom eaten.

(1) *Fat (Ghee)* —Ghee so called is commonly used but is often adulterated with *Vanaspati* (vegetable oil). The degree of adulteration may vary from 5 per cent to 90 per cent unless definite information regarding the origin of the ghee used is obtained the amount of vitamin D has been considered as very slight.

(Oil) —Different kinds of vegetable oil are used, mustard, linseed, cotton seed, rape-seed, arachis, sesame but none of these oils contain any vitamin D.

(9) Subsequent records. X-ray treatment and progress.

In addition the family history and the condition of other inmates of same house was carefully investigated in certain cases.

The constituents of the diets have been considered in detail in order to emphasize the necessity for accurate assessment.

For the purpose of correlating symptoms with diet, the diets have been classified as follows —

A	Excess cereal	1	Not neutralized by vitamin D in diet or sun
		2	Partially neutralized by vitamin D, in diet or sun or both. Some vitamin C
		3	Fairly adequately neutralized by vitamin D, in diet or sun or both. Vitamin C
B	Normal amount cereal	1	As (1) above
		2	As (2) above
		3	As (3) above
C	Diets deficient in quantity as well as in quality	1	As (1) above
		2	As (2) above
D	Border line diets		

Except in the diets C1 and C2, the intake of calcium and phosphorus was adequate.

It is recognized that with regard to the correlation of diet and clinical symptoms, many factors must be taken into account.

Diminution of symptoms may be caused by a combination of the following factors —Diet improved with regard to vitamins C and D, sun treatment, cod-liver oil, massage, alterations of housing conditions.

Exacerbation may be due to growth, pregnancy, lactation, malaria and other febrile and pathological conditions, alterations of housing conditions with lack of sunlight, poverty.

#### SEX INCIDENCE

The sex distribution of late rickets and osteomalacia has already been discussed(9) and it has been pointed out that hitherto observers in India have only recorded cases among women and girls, although the history of male cases recorded in other parts of the world would suggest that under certain conditions, e.g., hunger osteomalacia, florid cases might be expected among Indian men and boys also. Under normal Indian conditions, it is recognized that the men and boys of a family obtain adequate sunlight and since the usual custom is for them to be served first by the women of their household, the best of the available diet also, so that symptoms, if any, would be slight and not easily recognized.

## EXPERIMENTAL RESULTS

The results about to be described are derived from an examination of 265 female cases. They are classified according to district, religion and marriage as follows —

	Simla	Lahore	Amritsar	Kangra Valley	Total
Hindus, married	27	26	28	30	111
Mohammedans, married	26	18	11	2	57
Sikhs, married	1	7	8		16
Hindus, unmarried	9	24	5	5	43
Mohammedans, un-married	10	12	8	1	31
Sikhs, unmarried	2	2	3		7
TOTAL	75	89	63	38	265

Our first purpose is to examine whether lack of vitamin D either in the diet or due to want of sunlight or both is a predominant factor in the aetiology of late rickets and osteomalacia. If this is so we shall expect to find that the cases, having A1, B1 and C1 diets in which both vitamins C and D are lacking will exhibit major clinical symptoms as compared with those on diets A2, B2, C, and that patients receiving diets A3 and B3 will exhibit relatively slight clinical symptoms. A study of Table I, *a b, c* (i.e., total married, total unmarried and total of all cases), indicates this definitely.

TABLE I  
*Lack of vitamin D*

Diet	CLINICAL SYMPTOMS +++		CLINICAL SYMPTOMS ++		CLINICAL SYMPTOMS +		Total number of cases
	Actual number	Per cent	Actual number	Per cent	Actual number	Per cent	
(a) Total married cases—Hindus, Mohammedans and Sikhs							
A <sub>1</sub> , B <sub>1</sub> , C <sub>1</sub> , i.e., absolute lack of vitamin D	27	42	33	51	4	7	64
A, B, C i.e., partial lack of vitamin D	11	10	49	47	41	43	101
A <sub>3</sub> , B <sub>3</sub> , i.e., fairly adequately compensated for vitamins C and D	3	21	2	14	9	65	14
D, i.e., border line diets			2	40	3	60	5
stew							
(h) Total					Total		184

TABLE I—*contd*

Diet	CLINICAL SYMPTOMS +++		CLINICAL SYMPTOMS ++		CLINICAL SYMPTOMS +		Total number of cases
	Actual number	Per cent	Actual number	Per cent	Actual number	Per cent	
(b) Total married cases—Hindus Mohammedans and Sikhs							
A, B <sub>1</sub> , C <sub>1</sub>	2	9	11	52	8	39	21
A, B <sub>2</sub> , C	3	5	21	38	30	57	54
A, B <sub>2</sub>					6	100	6
Total							81
(c) Total married and unmarried cases							
A, B <sub>1</sub> , C <sub>1</sub>	29	34	44	51	12	15	85
A, B <sub>2</sub> , C <sub>2</sub>	14	9	70	45	71	46	155
A, B <sub>2</sub>	3	15	2	10	15	75	20
D			2	40	3	60	5
Total							265

In order to show that want of sun and of vitamin D in the diet are both intimately concerned with the ætiology of this disease, the Hindu, Mohammedan and Sikh cases have been arranged in a separate Table II

TABLE II

*Late rickets and osteomalacia, cases from 10 to 55 years of age*

	CLINICAL SYMPTOMS +++		CLINICAL SYMPTOMS ++		CLINICAL SYMPTOMS +		Total number of cases
	Actual number	Per cent	Actual number	Per cent	Actual number	Per cent	
Hindus, married	20	18	61	55	30	27	111
Mohammedans married	19	33.3	19	33.3	19	33.3	57
Sikhs, married	2	12	3	18	11	70	16
Hindus, unmarried	4	9	16	37	23	54	43
Mohammedans unmarried	1	3	15	48.5	15	48.5	31
Sikhs, unmarried			1	14.3	6	85.7	7

The Hindus may have a small quantity of milk in their diet, they do not observe purdah, but tend to derive such vitamin D as they get mainly from exposure to the sun. The housing conditions in the crowded bazaars are, however, such as afford scant opportunity for obtaining much sunlight, and the Hindu girl or woman rarely ventures any distance from her home. Mohammedan girls begin to observe purdah from 7 to 10 years of age onwards and thenceforth have even less opportunity of obtaining vitamin D from the sun, thus though the diet of the Mohammedans is rather more balanced than that of the Hindus, their mode of living leads to even more deficiency in vitamin D. On referring to Table II, this fact becomes evident. These statistics confirm very definitely the findings of former investigators with regard to the ætiology of osteomalacia (*see* references above).

#### HUNGER OSTEOMALACIA

Owing to the poverty of many of the inhabitants of India the diet is often not only defective from a qualitative but also from a quantitative point of view. Reference has already been made to the hunger osteomalacia prevalent in Germany and Austria as a result of post-war economic and social conditions. A reference to Table I, *a*, *b*, *c* (C1, C2 diets), totals married, unmarried and both, affords evidence of the fact that with what we might call 'ordinary' osteomalacia there is also associated the condition of hunger osteomalacia. The diets classified as C1 and C2 are such as would tend to result in the onset of hunger osteomalacia.

In the upper part of the Kangra Valley cases of definite osteomalacia and late rickets are met with among men and boys as well as among women and girls, which appear to resemble the hunger osteomalacia described by workers in Germany and Austria. In cases of great poverty among females in cities hunger osteomalacia may also occur.

TABLE III

*Incidence of C1 and C2 diets (deficient in quantity as well as quality)*

	Total number of cases	Number of cases with C <sub>1</sub> and C <sub>2</sub> diets
Simla	75	14
Lahore	89	21
Amritsar	63	14
Kangra Valley	38	—
TOTAL	265	60

#### EXCESS CEREAL

In considering the effect of an excess of cereal in the diet, the excess cereal diets (A1, A2, A3) and normal cereal diets (B1, B2, B3) have been compared with respect to clinical symptoms.

TABLE IV  
*Excess cereal diet Total married and unmarried cases*

Diet	CLINICAL SYMPTOMS +++		CLINICAL SYMPTOMS ++		CLINICAL SYMPTOMS +		Total number of cases
	Actual number	Per cent	Actual number	Per cent	Actual number	Per cent	
A <sub>1</sub>	13	39	18	55	2	6	33
B <sub>1</sub>	10	36	12	43	6	21	28
A <sub>2</sub>	4	8	24	48	22	44	50
B <sub>2</sub>	6	10	32	48	28	42	66
A <sub>3</sub>	3	20	2	13	10	67	15
B <sub>3</sub>					8	100	8
TOTAL							200

From the nature of the classification of the diets it is to be expected that A1 and B1, where vitamin D is absolutely deficient should show greater differences (if any) than A2 and B2 since within this latter classification, there will be considerable variation in amount of vitamin D, also it is to be expected that A3 and B3 should show differences greater than A2 and B2. The number of cases falling into each group is not large, but the evidence of the total number of cases suggests that the excess cereal factor does play some part in rendering the condition of the patient worse. Again comparing A3 and B3 diets in the same tables, it will be seen that patients on the A3 group of diet have shown worse clinical symptoms than those on B3 diets, with regard to A2 and B2 diets where the amount of vitamin D is variable, the correlation is less good.

#### FAMILY HISTORY

It has often been said that lack of vitamin D cannot afford a complete explanation of the aetiology of late rickets and osteomalacia and that other unknown factors must also play a large rôle. The chief argument in favour of such a statement has been that familial histories are very rarely obtained. Note has already been made of the very scanty references in the literature to familial tendencies in late rickets and osteomalacia.

In order to satisfy ourselves as to the health of control cases living in the same household as our patients we made careful inquiries in a series of cases



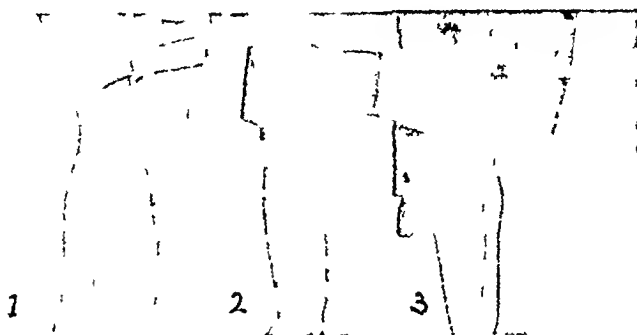
in Simla These family histories were obtained by asking patients to bring the other members of their household to the Clinic Thus we were able to observe that different members of the same family, inhabitants of the same household, having the same diet and being under the same housing conditions exhibited early rickets, late rickets or osteomalacia in varying degrees of severity Familial histories in other districts refer only to cases which came of themselves to the Clinic for treatment without any special inquiry

TABLE V

*Cases of osteomalacia, late rickets and early rickets, family histories of inhabitants of the same house*

Number in family	Station or area	Ages of patients	Relationship
5	Simla	36, 35, 27, 12	Mother, daughter, sister of mother, daughter-in-law
4	"	30, 7, 9, 11	Mother and daughters
4	"	28, 20, 17, 11	Mother, daughters and sister-in-law
4	"	30, 13, 10, 4	Mother and daughters
3	"	21, 22, 4	Mother, daughter, and cousin
3	"	50, 27, 4	Mother, daughter, grandchild
2	"	12, 14	Brother, sister
2	"	20, 12	Stepmother, daughter
2	"	30, 12	Mother, daughter
2	"	21, 3	Mother, daughter
2	"	22, 30	Sisters-in-law
3	Amritsar	40, 22, 13	Sisters, aunt
2	"	22, 14	Sisters
2	"	25, 20	Sisters
3	Lahore	22, 18, 8	Sisters and daughter
2	"	28, 1½	Mother, daughter
2	"	12, 5	Sisters
2	"	22, 10	Sisters
2	Kangra Valley	30, 25	Mother and son
2	"	50, 24	Mother, daughter

The evidence brought forward therefore, suggests that diet and housing conditions (i.e., the amount of sun available) are the chief causes of the familial tendency



*Photograph of 3 sisters*

1 Et 11 years 2 Et 9 years 3 Et 7 years  
All show deformities of legs (late rickets) No 3 shows comparatively greater deformity than No 2, because of more rapid rate of growth The mother had marked osteomalacia (confirmed by X-ray examination)

#### DISCUSSION AND CONCLUSIONS

This section (No III) of the osteomalacia inquiry is solely concerned with dietary factors The experimental results apart from the very definite corroboration of previous work that lack of vitamin D is the predominant factor in the aetiology of late rickets and osteomalacia, chiefly indicate the necessity for further inquiry along dietetic lines by means of animal experiments

In Western countries the disease is only sporadic but attention has been drawn to the fact that possibly in Western countries also there may be more osteomalacia than has been recognized hitherto Young(25) in an article on 'The woman damaged by child-bearing' draws attention to the frequent history of ill-health of the mother after child-birth, attributing this to damage done to the pelvis at the last pregnancy, which damage may have been due to a defective calcium metabolism causing osteoporoses and probably a very mild and hardly recognizable osteomalacia

In drawing together the results of this investigation the importance of the sun as a source of vitamin D in India must be emphasized, for Hindus because the vegetarian diet imposed by their creed affords little vitamin D content, and for Mohammedans because they exclude sun from their lives by observing purdah This practice of purdah is deeply to be deplored, since the greater severity of the disease among this particular group of the community is direct evidence of the need for ample sunlight to compensate dietic deficiencies It is interesting to note in this connection an observation made by one of our patients 'If she did not sit in the sun regularly her pains became worse again' Once the disease

becomes established, movement is extremely painful. The patient, therefore, necessarily tends to avoid movement, in other words she stays at home, and under the existing housing conditions this as a rule infers lack of sun, want of sun will cause a further exacerbation of the symptoms, and thus a vicious circle becomes established. It is interesting to note that the incidence of osteomalacia among the Sikhs, city women living in Amritsar and Lahore, although perhaps in the same proportion as that among Hindus and Mohammedans is less severe in character. This is probably due to the fact that many of them are accustomed to sitting in the sun on the roofs of their houses for some hours each day.

With regard to the question of hunger osteomalacia raised in connection with the diet where not only is the vitamin content insufficient but the calcium and phosphorus and all other constituents are also quantitatively deficient, the findings indicate that some of our cases might be classified under this heading. The greatest percentage of such cases are found in the upper part of the Kangra Valley, the condition, we would suggest, being strictly comparable to the post-war hunger osteomalacia in Central Europe. It is in this region that cases of late rickets and osteomalacia occur also among men and boys, the symptoms here are not so severe because a fair number of the patients work in the tea gardens and therefore get some vitamin D from the sun.

The importance of the amount of cereal in the diet still needs further investigation. In view of the difficulties of obtaining information from patients, the only method available appears to be individual personal investigation of the home conditions. The indications of this part of the investigation, however, point towards the large bulk of cereal in the diet causing an augmentation of the symptoms of the disease and possibly in some cases being the determining factor in its development.

It is proposed to investigate the rachito-genic action of the different cereals grown in the Punjab on rats, using the rat technique devised by Green and Mellanby (26).

There is yet another aspect of the dietary factors to be worked out—the possible relationship of vitamin C in connection with this disease. The diets of these patients are lacking in vitamin C almost as much as in vitamin D, the amount of fresh fruit and uncooked vegetables consumed is very slight. It is an empirical fact that the clinical symptoms do not clear up unless the diet includes fresh food. Whether this is due to lack of vitamin C or to lack of the proper acid-base ratio, or to lack of proper salt balance yet remains to be determined.

It may possibly be argued that the inquiry into family history is too scant to warrant much consideration, and that not enough attention has been paid to members of the same families who are free from clinical symptoms of rickets and osteomalacia. Circumstances have so far not permitted as full an inquiry as would be desirable, but from Table IV we venture to put forward the suggestion that a probable explanation of the familial tendency is due to a dietetic cause, or defective housing with consequent lack of sun, or both.

## SUMMARY

Previous observations go to prove that in spite of geographical or social differences osteomalacia and rickets are liable to occur whenever certain vitamins are deficient, either in the diet or from lack of sun. In the present paper the diets and housing conditions have been investigated of 265 cases of osteomalacia (late rickets) from four districts in the Punjab—Amritsar, Lahore, Simla and the upper part of the Kangra Valley where the disease is prevalent.

The results show that —

1 A consistent lack of vitamin D either in the diet or due to want of sunlight was found to be a predominant factor in the aetiology, confirming the experimental findings in other parts of the world. The importance of the sun as a source of vitamin D in India is emphasized both for the Hindu population because their diet is poor in vitamin D, and for the Mohammedans because of the greater severity of the disease found among those women who observe purdah. The amount of calcium and phosphorus in the patients' diet was usually adequate.

2 Cases of late rickets and osteomalacia are recorded among men and boys as well as among women, comparable to the post-war hunger osteomalacia in Central Europe. Most of these cases are found in the upper part of the Kangra Valley where not only is the vitamin content of the diet insufficient, but the calcium and phosphorus and all other constituents are quantitatively deficient also.

3 Excess cereal in the diet plays some part in rendering the condition of the patient worse.

4 Clinically symptoms do not clear up unless the diet includes fresh food, whether this is due to a previous lack of vitamin C or of the proper acid-base ratio, or of proper salt balance yet remains to be determined.

5 In a series of cases the occurrence of osteomalacia, late rickets and early rickets among members of the same household living under similar conditions, is suggestive of familial tendencies being due either to a dietetic cause or to defective housing (i.e., lack of sun).

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# OSTEOMALACIA (LATE RICKETS) STUDIES

## Part IV

### A PRELIMINARY NOTE ON THE INCIDENCE OF RICKETS AND DENTAL CARIES AMONG SCHOOL CHILDREN IN INDIA

BY

DAGMAR CURIEL WILSON, M D (Glas ), D P H (Camb ), W M S (Retd ),  
*In charge Osteomalacia Inquiry Indian Research Fund Association,*

AND

ELLA SURIE, M Sc (Lond ),  
*Professor of Physiology Lady Hardinge Medical College, New Delhi, working  
under the Indian Research Fund Association*

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An opportunity has occurred of observing the relation of the incidence of dental caries to rickets during the course of an inquiry into dietary factors in the ætiology of Osteomalacia(1)

Though it is still frequently stated in textbooks that Eastern races rarely suffer from dental caries, the fact that dental caries is becoming more prevalent is gradually being recognized. Frampton Badcock(2) in his appeal for 'an indigenous profession of dental surgery in India' at first states 'dental caries, the scourge of the Occident, is comparatively rare,' but subsequently in the same article says, 'India, however, is no longer immune from Western civilization and its effects are evidenced by change in habits, customs and diet. Change of diet, especially the increased consumption of flesh foods and alcohol, is mainly responsible for the introduction, especially into the crowded cities, of dental caries.' The amount of dental caries is undoubtedly increasing, although the authors do not agree with the writer of this article as to the causative factors.

The 100 children available for this investigation were all suffering from rickets of varying degrees of severity and were selected, as far as possible, as being representative of different ages (5 to 17 years of age)

The classification of the severity of rickets is based on clinical and X-ray evidence, and that of dental caries and hypoplasia determined by means of oral examination. The examination of children of ages varying from 5 to 17 must include consideration of dental caries and hypoplasia in both the deciduous and permanent teeth. The amount of dental caries and hypoplasia in the deciduous teeth is very considerable. Gross caries is usually to be found in the pre-molars, some caries in the remaining teeth, whereas hypoplasia is widespread. The permanent teeth except for the first molar, exhibit much less caries and hypoplasia, both of which are also less widespread. Calcification in the first molar is beginning just after birth and therefore this tooth will tend to reflect any defects in the deposition of calcium during the first years of life. In this note the dental caries and hypoplasia in the deciduous and permanent teeth have been grouped together.

The authors are well aware that many clinical conditions other than rickets may supervene and predispose to dental caries, for this reason the health record of each case has been investigated.

A summary of the results obtained is printed in the following Table —

TABLE  
Total number of cases examined—100

Group	Cases	Number	Caries per cent	Hypoplasia per cent
1	Gross rickets	9	100	100
2	Rickets	24	79	96
3	Mild rickets	53	74	90
4	Almost normal	14	21	85

*Analysis of the Table*

Group 1 Severe rickets Total number of cases 9

Per cent of  
cases

Per cent of  
cases

Gross caries  
Caries  
Mild caries  
No caries

Gross Hypoplasia  
Hypoplasia  
Mild Hypoplasia  
No Hypoplasia

89  
11

Group 2 Rickets Total number of cases 24

Gross caries

21

Gross Hypoplasia

59

42

Hypoplasia

33

Mild caries

16

Mild Hypoplasia

4

No caries

21

No Hypoplasia

4

Group 3	Mild rickets	Total number of cases	53
Gross caries	2	Gross Hypoplasia	15
Caries	17	Hypoplasia	28
Mild caries	55	Mild Hypoplasia	47
No caries	26	No Hypoplasia	10
Group 4	Almost normal	Total number of cases	14
Gross caries		Gross Hypoplasia	7
Caries	7	Hypoplasia	14
Mild caries	29	Mild Hypoplasia	64
No caries	64	No Hypoplasia	15

It will be observed that the 9 cases of severe rickets all show gross dental caries, and in 89 per cent of the cases are accompanied by gross hypoplasia. In the 24 cases exhibiting rickets, the percentages of gross caries and gross hypoplasia have decreased, while those of caries and hypoplasia on the other hand have increased. Some of the cases exhibit no caries and some no hypoplasia, but in no instance was there simultaneous absence of both. Finally the greater majority of cases (53 in number) classified as mild rickets show a further decrease of caries and hypoplasia, but an increase in mild caries and mild hypoplasia as also an increase in the number of cases having neither caries nor hypoplasia.

These figures although few and of a preliminary nature, are indicative of the fact that the incidence of rickets and caries in parallel degrees of severity are co-existent. Since rickets is now recognized as a deficiency disease, the dietary factor in the etiology of dental caries must also be of primary importance.

This conclusion is not new, it substantiates the work of May Mellanby in England, the exponent of this theory.

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# THE DIAGNOSIS OF KALA-AZAR BY THE UREA STIBAMINE TEST

BY

MAJOR H E SHORTT, FZS, IMS,

MAJOR A C CRAIGHEAD, IMS,

ASSISTANT SURGEON R O A SMITH, DTM, IMD,

ASSISTANT SURGEON H A H D'SILVA, MRCS, LRCP, IMD,

AND

SUB ASSISTANT SURGEON SRIBAS DAS

(*Kala-azar Commission*)

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THE clinical features of kala-azar are so similar to those of other long-continued fevers in the tropics that a simple test to diagnose the condition has been a much-felt want. The aldehyde and globulin precipitation tests, while useful in well-established cases of kala-azar, yield no certain information in early cases and recourse must then be had to spleen or liver puncture. While this, in experienced hands, is a delicate and definite means of diagnosis, especially when supplemented by cultural methods, the general practitioner rightly hesitates to resort to it.

In June 1927 Chopra, Gupta, and David published a preliminary note on a new test discovered by them at Calcutta. It was claimed by them that this test gave reliable indications even in the early stages of kala-azar and it was this claim which led us to undertake an independent investigation of the test.

Chopra and his collaborators found that the mixture of solutions of antimony compounds, especially those containing urea, with the serum of cases of kala-azar resulted in the production of a thick flocculent precipitate, while the sera of other subjects produced no such reaction. These effects were said to be produced even in early cases of kala-azar.

## TECHNIQUE ADOPTED IN THE PRESENT INVESTIGATION

*Collection of serum*—The serum was collected in Wright's capsules from patients attending the Kala-azar Commission Clinic for the diagnosis and treatment

of kala-azar, and was stored overnight at 37°C during the winter months and at room temperature during the hot weather

*Diagnosis of kala-azar* —The routine method of diagnosis of kala-azar carried out by the Kala-azar Commission is liver or spleen puncture and direct microscopical examination of the material obtained. Where necessary, this is supplemented by cultural examination of the puncture material. Examination of the peripheral blood for parasites is also done as a routine measure.

In the series of cases the subject of this investigation cultures were made in 288 instances as a special precaution against error in giving a negative diagnosis. In only one case was a positive culture obtained where the result of the direct microscopic examination was negative. It may, therefore, be taken for granted that the margin of error in diagnosis was negligible.

*Dilutions of urea stibamine and serum* —The first 200 tests were performed with undiluted serum and a 4 per cent solution of urea stibamine in distilled water, equal parts of each being used in each test. This series will, in the following account, be referred to as A series.

The next 24 tests were performed with serum diluted nine times with distilled water (1 in 10) and 4 per cent solution of urea stibamine in distilled water. These cases constitute B series in the account.

The remaining 476 tests were performed with serum diluted nine times with distilled water (1 in 10) and a 2 per cent solution of urea stibamine in distilled water. These cases constitute C series in the account.

*Method of performing the test* —A small quantity (0.5 c.c.) of the serum, or diluted serum, is taken in a Dreyer's tube. To this an equal volume of the urea stibamine solution is added carefully so as to form a layer on top of the serum.

*Reading of the test* —This was done within 5 to 20 minutes of putting up the tests. We recognized no degrees of positivity or negativity. If definite flocculation appeared at the junction of the two liquids, the test was considered positive. If only cloudiness appeared, or if the mixture remained clear, the test was recorded as negative.

#### RESULTS OF THE TESTS

##### *A Series*

In this series of 200 tests the test was done with undiluted serum and 4 per cent solution of urea stibamine in distilled water. The findings are recorded below in tabular form. For purposes of comparison of the test with the findings obtained by the more precise methods of liver or spleen puncture, the agreement or disagreement of the tests with the diagnosis obtained by puncture is shown —

UREA STIBAMINE TEST AND DIAGNOSIS BY SPLEEN OR LIVER PUNCTURE AGREE.		UREA STIBAMINE TEST AND DIAGNOSIS BY SPLEEN OR LIVER PUNCTURE DISAGREE.	
Positive	Negative	Test positive Puncture negative	Test negative Puncture positive
20	64	116	0

## COMMENTS ON THE FINDINGS IN A SERIES

*Malaria*—Of the 200 tests 28 were cases of malaria and these gave the following results—

UREA STIBAMINE TEST AND DIAGNOSIS BY SPLEEN OR LIVER PUNCTURE AGREE		UREA STIBAMINE TEST AND DIAGNOSIS BY SPLEEN OR LIVER PUNCTURE DISAGREE	
Positive	Negative	Test positive Puncture negative	Test negative Puncture positive
0	12	16	0

Of the 16 malaria cases giving a positive test—

4 were benign tertian malaria

4 were malignant tertian malaria

7 were quartan malaria

1 was benign tertian and malignant tertian malaria

Of the 12 malaria cases giving a negative test—

4 were benign tertian malaria

6 were malignant tertian malaria

1 was quartan malaria

1 was benign tertian and malignant tertian malaria

*Precious treatment*—Among the 200 tests, 30 patients had had previous treatment for kala-azar at various treatment centres operating in the district and the majority were probably not yet cured. Of these cases 2 still showed kala-azar parasites in the puncture material and one had in addition dermal lesions. Both gave a positive test. In the remaining 28 cases 23 gave a positive and 5 a negative test. In the 23 cases giving a positive test malaria parasites were found in the blood of 7.

*Healthy adults*—A series of 12 tests performed on healthy adults gave uniformly negative results.

As the general results of the tests were so much in disagreement with the laboratory findings, the next series of tests was commenced.

*B Series*

In this series of 24 tests, the test was done with serum diluted 9 times (1 in 10) and 4 per cent solution of urea stibamine in distilled water. The findings are recorded below in tabular form—

UREA STIBAMINE TEST AND DIAGNOSIS BY SPLEEN OR LIVER PUNCTURE AGREE		UREA STIBAMINE TEST AND DIAGNOSIS BY SPLEEN OR LIVER PUNCTURE DISAGREE	
Positive	Negative	Test positive Puncture negative	Test negative Puncture positive
6	6	12	0

As the results here recorded still showed 50 per cent of disagreement with the laboratory findings, the next series of tests in which a higher dilution of urea stibamine was utilized was undertaken

### C Series

In this series of 476 tests, the test was done with serum diluted 9 times (1 in 10) and 2 per cent solution of urea stibamine in distilled water. The findings are recorded below in tabular form —

UREA STIBAMINE TEST AND DIAGNOSIS BY SPLEEN OR LIVER PUNCTURE AGREE		UREA STIBAMINE TEST AND DIAGNOSIS BY SPLEEN OR LIVER PUNCTURE DISAGREE	
Positive	Negative	Test positive Puncture negative	Test negative Puncture positive
79	308	81	8

*Comments on the findings in C Series*—This series shows 81.3 per cent of agreement with laboratory findings, which still leaves a large margin of error.

Out of the 160 positive tests in this series, 79 were cases of kala-azar. Of the remaining 81 tests 19 were cases of malaria, one was filariasis, one liver abscess, and in 60 no parasite of any sort was demonstrated. Out of these latter 60 cases, 16 had previously received treatment for kala-azar and it may be assumed were, or had been, cases of kala-azar. Excluding these there still remain 65 positive tests which disagree with the laboratory findings, among which 21 were definitely diagnosed diseases other than kala-azar. This still leaves, in the case of the positive tests ( $160 - 16 = 144$ ), a rate of disagreement with the laboratory findings of 45.1 per cent.

*Cases in which the test was negative and puncture finding positive*—The 8 tests in which the test was negative and the puncture result positive, represent only 6 cases of kala-azar. Two of the cases were tested a second time to confirm the previous results. All these were early cases varying in duration up to at least two months. In two cases the spleen was not palpable and in the other four it measured from one to three finger-breadths below the costal margin.

*Malaria*—Of the 476 tests, 107 were cases of malaria and these gave the following results —

UREA STIBAMINE TEST AND DIAGNOSIS BY SPLEEN OR LIVER PUNCTURE AGREE		UREA STIBAMINE TEST AND DIAGNOSIS BY SPLEEN OR LIVER PUNCTURE DISAGREE	
Positive	Negative	Test positive Puncture negative	Test negative Puncture positive
0	87	19	0

Of the 19 malaria cases giving a positive test—

- 9 were benign tertian malaria
- 5 were malignant tertian malaria
- 4 were quartan malaria
- 1 was undetermined
- 1 was benign tertian and malignant tertian malaria

Of the 87 malaria cases giving a negative test—

- 27 were benign tertian malaria
- 29 were malignant tertian malaria
- 20 were quartan malaria
- 10 were undetermined
- 1 was benign tertian and malignant tertian malaria

*Cases other than kala-azar*—Of these there were 20 and a negative reaction was obtained in every instance. The series was distributed as follows —

- 1 case of cardiac disease
- 1 case of Naga sore
- 1 case of syphilis
- 1 case of beri-beri ( ? epidemic dropsy)
- 2 cases of asthma
- 12 healthy adults

2 cases which had had kala-azar but at the time showed no signs of disease

*Previous treatment*—Among the 476 tests 60 tests were performed on cases who had had treatment for kala-azar, and of whom many were probably not yet cured. Of these cases 7 still showed kala-azar parasites in the puncture material. In the remaining 53 cases, 19 gave a positive and 34 a negative test. In the 19 cases giving a positive test malaria parasites were found in the blood of 2.

### CONCLUSIONS

Although a total of 700 tests was performed, our conclusions are based only on the last 476 tests in our series for reasons which appear in the text. These conclusions are —

- 1 The test agreed with the protozoological findings in 81.3 per cent of instances
- 2 Out of 144 positive tests, the diagnosis of kala-azar would have been erroneous in 65 instances—an error of 45.1 per cent
- 3 Out of 314 negative tests, kala-azar parasites were found in 8 instances—an error of 2.5 per cent
- 4 The test is of more value in excluding kala-azar than in revealing its presence

Our thanks are due to Dr L. E. Napier, M.R.C.S., L.R.C.P., who, during the absence on leave of the senior author, acted as Director of the Kala-azar Commission and supervised the researches in progress.

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*PHLEBOTOMUS ARGENTIPES* CAUGHT IN NATURE  
INFECTED WITH *LEISHMANIA DONOVANI*.

BY

MAJOR H E SHORTT, F Z S, I M S,  
MAJOR A C CRAIGHEAD, I M S,  
ASSISTANT SURGEON R O A SMITH, I M D,

AND

C S SWAMINATH  
(*Kala-azar Commission*)

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In 1926 Shortt, Barraud and Craighead recorded the first *Phlebotomus argentipes* caught in nature infected with *Leishmania donovani*, since when no further records of similar findings in nature have been published. We have therefore made some effort to amplify our work in this field by the dissection, as opportunity offered, of flies obtained in nature in Assam from the houses of kala-azar cases.

The flies were collected at intervals over the period 28th March, 1928 to 30th August, 1928. In all cases only houses known to contain a kala-azar case were searched for *P. argentipes* and, in most instances, each house was searched on several occasions, the number of examinations per house varying from one to eight. The small number of flies dissected is due chiefly to the fact that only a few specimens are, as a rule, caught at any one visit and the opportunities at our disposal for this aspect of our researches were limited. At the same time it will be evident from the results that a certain percentage of infected flies must always be present in houses harbouring kala-azar cases, a conclusion in harmony with findings in the laboratory when clean *P. argentipes* are fed upon cases of kala-azar. The results of the dissections are given in a tabular form (*see opposite*).

CONCLUSION

Out of 226 *P. argentipes* collected in Assam during the period 28th March, 1928 to 30th August, 1928 from houses of kala-azar patients, 7 proved to be infected with *L. donovani*.



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We desire to thank Dr. L. E. Napier, M.R.C.S., L.R.C.P., for his general supervision of the researches of the Kala-azar Commission during the absence on leave of the senior author for whom he acted as Director.

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# PRELIMINARY TRANSMISSION EXPERIMENTS IN INDIAN KALA-AZAR NOT INVOLVING THE USE OF AN INTERMEDIATE VECTOR

BY

MAJOR H E SHORTT, F R S, I M S,

MAJOR A C CRAIGHEAD, I M S,

ASSISTANT SURGEON R O A SMITH, D T M, I M D,

AND

C S SWAMINATH

(*Kala-azar Commission*)

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As a result of the uniformly negative results obtained by all workers in attempts to prove the hypothesis of *Phlebotomus*-transmission of kala-azar, and encouraged by the success obtained by various workers, including ourselves, in infecting animals by the oral route, we have considered necessary the initiation of experiments to test means of transmission of kala-azar other than those involving the aid of some blood-sucking intermediary

With this aim in view, we devised the three series of experiments described below. Owing to the small number of Chinese hamsters (*Cricetus griseus*) at our disposal, these experiments are only in the nature of a preliminary feeling of our way and it is hoped will be so regarded until they can be amplified when further supplies of suitable experimental animals are available

## *Series I Proximity experiments*

Adelheim (1924) has recorded an experiment in which a mouse infected with kala-azar and placed in a jar along with an uninfected mouse resulted in the infection of the latter. If the hypothesis of direct transmission of kala-azar by contaminative means be considered a possibility, it will be evident that an experiment conducted on such lines, if continued for a sufficiently long time, will give ample opportunity for the healthy animal inadvertently to ingest any excretory products of the infected animal which may contain infective material in the shape of *Leishmania* parasites. In using hamsters, the simple form of experiment adopted by Adelheim is seldom feasible. Two hamsters placed together, and especially if of different sexes, almost invariably fight, a disagreement usually resulting in the death of the weaker animal. To avoid this termination of the

experiment, we placed each hamster in a rectangular wire cage. Two cages, one containing an infected and one a healthy animal, were connected by adjacent sides with wire fastenings and were placed together in a larger earthenware vessel made to take two cages so connected. Six infected and six uninfected hamsters (*Cricetulus giseus*) were used in the experiment, which was commenced on 27th April, 1928. The infected hamsters had previously been inoculated intra-peritoneally from another infected hamster and were heavily parasitized.

Owing to a misunderstanding, the experiment was not carried on quite on the lines originally intended. The intention was to have the cages enclosed in a larger vessel of non-porous material which would prevent the absorption of any infective excreta and urine but, actually, more or less porous earthenware was used. In addition, as the cages were intentionally never cleaned, the accumulation of the daily food material in the form of gram, which dropped through the meshes of the wire cages, resulted in the gradual raising of the cages above the floor of the containing receptacle until they were several inches above their original level. This gave little opportunity for the excreta and urine of one animal to contaminate the cage of the other, since any liquid material at once percolated down through the accumulated food debris at the site where it was deposited. From these considerations we believe that only the early stages of the experiment, before the excessive accumulation of food material, were in the nature of a fair test, and that the later stages were necessarily ineffective. This would mean that the healthy animals could only ingest infective material early in the course of the experiment or not at all, and strength is given to this view by the fact that the two previously healthy animals which became infected each showed a very heavy infection indicating a duration of many months. The results of this series of experiments are given below in tabular form —

TABLE I  
Showing details of 'proximity' experiments

Originally infected animal	Originally healthy animal	Date placed together	Date of examination of originally healthy animals.	Result of examination	REMARKS
Hamster A	Hamster A 1	27-4-28	29-4-29	Negative	Hamster A died on 5-4-29
" G C	" G 1 " C 1	"	10-5-29 29-4-29	Negative Positive Very heavy infection	Hamster C died on 5-4-29
" D E	" D 1 " E 1	"	10-5-29 10-5-29	Negative Negative	
" 25C	" 25C 1	"	29-4-29	Positive Very heavy infection	Hamster 25C died on 21-7-28

*Remarks on Table I*

It will be seen from the 'remarks' column that three of the originally infected hamsters died before the date of examination of the originally healthy animals. One of the former (25C) died not more than three months after the animals had been placed together and, as its opposite number proved later to be one of the two infected, it follows that the infection must have taken place in the earlier stages of the experiment. This strengthens the conclusion previously come to on other grounds that both the animals infected must have acquired their infection at a comparatively early stage before the excessive accumulation of food interfered with the conditions necessary to the continued effectiveness of the experiment.

The two animals which acquired infections were very heavily parasitized, the spleen in each case being enlarged to about fifteen times the normal size. In spite of the heavy infection, the animals remained otherwise perfectly healthy and well nourished.

*Series II Experiments with centrifuged urine*

It has been shown (Shortt, 1923, and Shortt, Swaminath and Sen, 1923) that a certain proportion of kala-azar patients excrete *Leishmania* in their urine and this finding has been confirmed in the case of hamsters (Young, 1927). This appeared to furnish obvious indications for the infection of healthy animals by the oral route and a series of experiments to bring about this result was devised. A series of twenty hamsters was given the centrifuged deposit from the urine of untreated cases of human kala-azar by the oral route. The method adopted was to allow a few drops of the centrifuged urine deposit to fall from a pipette into the open mouth of the hamster. The urine was never more than a few hours old and the administration was repeated at intervals of about a week or even less throughout more than a year. The details of these experiments are noted below in tabular form —

TABLE II  
*Showing details of urine deposit experiments*

Experimental animal	Nature of infective material	Date of commencement of experiment	Date and method of termination of experiment	Number of administrations of infective material	Results
Hamster A 1	Deposit of centrifuged urine from untreated kala-azar cases	12-1-28	Post-mortem 21-3-29	74	Negative
" A 2	"	"	"	68	"
" A 3	"	"	"	71	"
" A 4	"	"	"	69	"

TABLE II—*contd*

Experimental animal	Nature of infective material	Date of commencement of experiment	Date and method of termination of experiment	Number of administrations of infective material	Results
Hamster A 5	Deposit of centrifuged urine from untreated kala-azar cases	12-1-28	Post-mortem 21-3-29	68	Negative
" A 6	"	13-1-28	"	72	"
" A 7	"	"	"	72	"
" A 8	"	"	"	75	"
" A 9	"	12-1-28	Found dead on 8-8-28	34	"
" A 10	"	"	Post-mortem 21-3-29	65	"
" A 11	"	20-1-28	"	75	"
" A 12	"	"	"	74	"
" A 13	"	"	Found dead on 25-7-28	31	"
" A 14	"	"	Post-mortem 21-3-29	71	"
" A 15	"	"	"	71	"
" A 16	"	21-1-28	Post-mortem 5-7-28	31	"
" A 17	"	"	Post-mortem 21-3-29	62	"
" A 18	"	"	"	69	"
" A 19	"	"	Found dead on 6-9-28	38	"
" A 20	"	"	Post-mortem 21-3-29	68	"

*Remarks on Table II*

The failure of a series of twenty animals to show any infection after repeated feeds on the centrifuged urine of untreated kala-azar cases may be interpreted in a variety of ways which we do not propose to go into at the present stage of our investigations. Results obtained by us in cognate researches, however, would appear to indicate that the excretion of parasites in the urine of animals infected with kala-azar demands conditions other than the mere presence of parasites in the excretory organs. We hope to say more on this subject in a subsequent publication.

*SERIES III Experiments with centrifuged urine mixed with food materials*

This series of experiments was ancillary to those described in the previous series. The hamsters instead of being given urine deposit direct were fed upon gram which had mixed with it the deposits of centrifuged urine from untreated kala-azar cases. Twelve hamsters were utilized in the experiments and, as the same series of cases of kala-azar was used as in the previous series, the uniform absence of infections which resulted is not surprising. Details of the experiments are given below in tabular form —

TABLE III

*Showing details of experiments with gram mixed with urine deposits*

Experimental animal	Nature of infective material	Date of commencement of experiment	Date and method of termination of experiment	Number of administrations of infective material	RESULTS
Hamster C 1	Gram mixed with urine deposit	12-1-28	Found dead on 22-7-28	39	Negative
" C 2	"	"	Post-mortem 30-4-29	74	"
" C 3	"	"	Found dead on 15-6-28	32	"
" C 4	"	"	Post-mortem 30-4-29	74	"
" C 5	"	"	"	74	"
" C 6	"	"	Found dead on 29-10-28	55	"
" C 7	"	"	Found dead on 6-8-28	40	"
" C 8	"	"	Post-mortem 30-4-29	71	"
" C 9	"	"	"	72	"
" C 10	"	"	"	72	"
" C 11	"	"	Found dead on 5-10-28	51	"
" C 12	"	"	Found dead on 1-9-28	44	"

No remarks on this table are called for as those made on Table II are equally applicable here.

## SUMMARY

1 Out of six uninfected hamsters kept continuously in proximity to six infected hamsters two became heavily infected.

2 Out of twenty hamsters fed repeatedly on the deposit from the centrifuged urine of untreated kala-azar cases none became infected

3 Out of twelve hamsters fed on gram contaminated by the centrifuged deposit from the urine of the same series of kala-azar cases none became infected

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# THIRD SERIES OF TRANSMISSION EXPERIMENTS IN KALA-AZAR WITH *PHLEBOTOMUS ARGENTIPES*

BY

MAJOR H E SHORTT, FZS, IMS,  
MAJOR V C CRAIGHEAD, IMS,  
ASSISTANT SURGEON R O A SMITH, DTM, IMD,

AND

C S SWAMINATH  
(Kala-azar Commission)

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## *A Experiments with human volunteers and Phlebotomus argentipes*

THIS series of experiments was instituted to supplement those already carried out with human volunteers and reported on in a previous publication (Shortt, Craighead, Smith and Swaminath, 1928). It was felt that the four volunteers then made use of was an insufficient number on which to base any definite conclusions and it was therefore decided to carry out a further series of experiments using a fresh batch of volunteers and submitting them to the bites of infected *Phlebotomus argentipes* on a scale even more intensive than that previously achieved.

In the publication referred to we have given a detailed description of the precautions taken to avoid preventable fallacies in the experiment and it is only necessary to say that the conditions for the experiments there laid down were rigidly adhered to in the present series.

Seven male Khasi volunteers, none of whom had ever been in a kala-azar endemic area, were enlisted. Before the commencement of the experiment each was examined by cultural methods for the presence of kala-azar infection, although this was really an entirely unnecessary precaution since none of them had previously left Shillong, where kala-azar has never existed.

Full details of the experiments are given below in tabular form —



TABLE I

Showing details of transmission experiments with human volunteers and *Phlebotomus argentipes*

Volunteer	Date of commencement of experiment	Date of last exposure to bites of flies	Date of examination by liver puncture	Number of separate occasions on which bitten and largest number of flies fed on one occasion	Total number of flies fed	Number of flies known to be infected	Number of flies known to be not infected	Probable number of infected flies	Results
U Morris Brenlow	25-1-28	28-2-29	23-4-29	54/98	1,561	77	233	397	Negative
U Wilan Rees	30-1-28	1-3-29	"	48/219	2,049	88	230	520	"
U Niza	1-2-28	26-2-29	"	52/184	1,838	95	255	463	"
U Edren	3-2-28	26-4-29	No examination by liver puncture	9/95	230	17	36	58	Experiment discontinued on 26-4-28 as he was found to be suffering from pulmonary tuberculosis
U Muharell	6-2-28	21-2-29	23-4-29	48/141	1,687	72	234	429	Negative
U Mana	8-2-28	23-2-29	"	46/125	1,673	73	197	425	"
U Sivarvell	1-6-28	19-2-29		33/104	1,259	47	191	320	Negative Taken on in place of U Edren, after the latter was discharged

## Remarks on Table I

The flies used in the experiments were exclusively those having their third and subsequent feeds, as it seems unlikely that flies at an earlier stage can be infective. We have always stressed this point in our previous publications as it has seemed to us that many other workers on kala-azar were satisfied as to the possibility of infection by the fly whenever flagellates could be demonstrated in its gut by dissection after a feed. Although we do not deny this possibility, a knowledge of the anatomical structure of the fly would seem to render it extremely unlikely that a few flagellates in the mid-gut should obtain exit from the proboscis during its act of feeding. The heavy infections of the anterior parts of the alimentary canal seen in flies ready for their third feed would, however, present at least the possibility of a number of the flagellates gaining access to the wound produced by the proboscis of the fly.

The actual intensity of feeding by flies under the conditions of the experiment is immensely greater than would ever obtain under natural conditions in Assam. In addition to this the flies feeding in nature might have had their previous feeds on uninfected human beings or on animals, so that only a relatively small proportion would be infected with *Leishmania*, whereas all the flies used in the experiment had had at least their initial feed on an untreated case of kala-azar and certainly contained a very large proportion of infected flies. In other words the conditions of the experiment multiplied by many times the actual number of flies which might have fed on an individual in Assam and multiplied to a far greater extent the proportion of infected flies. The examination of the volunteers after the end of the period of feeding was by liver puncture and culture of the material obtained on NNN medium. At the time of writing, 18 months after the initiation of the experiments, none of the seven volunteers has shown any sign of infection with kala-azar, but the lapse of a considerable time will be required before it can be said that the results of the experiments have been definitely negative.

B Experiments with white mice and *Phlebotomus argentipes*

The mice used in these experiments were animals which were used for feeding infected flies upon on days when they were not required for human volunteers. As will be seen from the table the intensity of feeding was very great but, as in the experiments with human volunteers, the results were negative. Details are given below in tabular forms.

C Experiments with white mice and *Phlebotomus argentipes* fed on blood only

This small series of experiments was devised in order to see whether the failure to produce infections by the bites of *P. argentipes* was due to the fact that after their initial feed on a kala-azar case, being fed indifferently on other animals. It was considered a possibility that the blood of animals other than

TABLE II  
Showing details of transmission experiments with white mice and *Phlebotomus argentipes*

Experimental animal	Date of commencement of experiment	Date of last exposure to the bites of flies	Date on which the animal was sacrificed	Total number of flies	Number of flies known to be infected	Number of flies known to be not infected	Probable number of infected flies	Results	
								Cultural	Microscopical
Mouse 25	3-4-28	10-3-29	21-3-29	995	90	251	245	Negative	Negative
" 26	4-4-28	9-3-29	21-3-29	925	64	221	227	"	"
" 27	27-2-29	29-3-29	11-7-29	259	17	52	77	"	"
" 28	3-3-29	2-4-29	11-7-29	124	10	20	37	"	"
" 29	19-3-29	3-4-29	11-7-29	47	5	5	14	"	"
" 30	20-3-29	30-3-29	11-7-29	69	4	7	20	"	"

from whom the parasite was originally obtained by the sandfly, might interfere with its infectivity

To avoid this the flies in this series, after their initial feeds on an untreated case of kala-azar, were fed exclusively on human beings (the same case or other kala-azar cases) up to their fourth feed. The fifth and subsequent feeds were on the experimental mice. By this means the parasites present in the fly as a result of its initial feed were never exposed to other than human blood until their feed on the experimental animal. At the same time the fact that all the flies were having their fifth or subsequent feed ensured that the infection with flagellates was a comparatively old one and that sufficient time had elapsed for the production of special infective forms if any such exist. As some doubt seems to exist whether mice when infected retain their infection indefinitely, or tend towards spontaneous cure, the animals which were the subject of these experiments were sacrificed at a comparatively early date. The details of this series of experiments are given below in tabular form

#### *Comments on the three series of experiments*

It will be seen from the tables that the experiments had a duration of one year and must have involved an immense amount of laborious routine work. This will be realized when it is remembered that the flies used for feeding on human volunteers or experimental animals were exclusively those having their third or subsequent feed. Those used in Series C were those having their fifth or subsequent feed. This meant that each fly used had to be fed at least twice before it was of any use for experimental purposes. As the mortality among the flies after feeding is always considerable to obtain the number of flies actually used in the experiments, the numbers in the tables must be multiplied at least six times. As an indication of the amount of entomological material necessary to the carrying out of these experiments, we give below a few details of the numbers of flies bred out in the laboratory and used for feeding purposes

Number of flies bred out during the duration of experiments 273,467

Number of flies fed in the laboratory during the duration of the experiment 79,939

These remarks have been made in order to emphasize the intensive nature of the feeding by flies to which all the subjects of experiment were exposed. As has previously been remarked, this was enormously in excess of what could occur under the most favourable circumstances imaginable under natural conditions in Assam. The failure of any of the subjects of experiment to show infection with kala-azar as a result while fresh infections were daily coming for diagnosis from the surrounding town and country-side, is very difficult of explanation if the theory of *Phlebotomus* transmission is to be maintained. We can only suppose that some essential factor in the process of infection has been omitted in our experiments which is present under natural conditions, or that the vast amount of labour expended by us under the trying climatic conditions of Assam during a

TABLE III.

Showing details of transmission experiments with white mice and *Phlebotomus argentipes* fed on human blood only

Experimental animal	Date of commencement of experiment	Date of last exposure to the bites of flies	Date on which the animal was sacrificed	Total number of flies fed	Number of flies known to be infected	Number of flies known to be not infected	Probable number of infected flies	RESULTS	
								Cultural	Microscopical
Mouse X	8-5-29	25-5-29	11-7-29	20	1	10	4	Negative	Negative
"	7-5-29	16-5-29	11-7-29	17	2	5	4	"	"
"	9-5-29	26-5-29	11-7-29	27	3	7	6	"	"

period of five years has been expended on an insect which is not an essential link in the chain of infection

#### ACKNOWLEDGMENTS

We desire gratefully to acknowledge the services of Dr L E Napier, M R C S I K C P who during the absence of the senior author on leave, officiated as Director of the Kala-azar Commission and exercised a general supervision over the experiments in progress

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# AN INVESTIGATION OF SAMPLES OF RICE BELIEVED TO HAVE BEEN THE CAUSE OF BERI-BERI IN BURMA

BY

MAJOR G. VERGHESE, I M S.,

*District Laboratory, Maymyo*

## INTRODUCTION

FOLLOWING up, in certain respects, the work of Acton and Chopra (1925), an investigation was carried out at the District Laboratory, Maymyo, on samples of rice believed to have been the cause of cases of beri-beri in Burma. The investigation was entirely from the point of view of possible infection by micro-organisms.

The line of work consisted of —

- 1 (a) Obtaining samples of rice consumed by beri-beri cases, and to study their special characteristics  
(b) Isolation and culture of organisms, aerobic and anaerobic, from these grains  
(c) Determination of the toxicity or non-toxicity of the organisms isolated
- 2 Feeding experiments on animals, using a basal diet with variations of healthy and infected rice. Observation and record of symptoms produced.
- 3 Examination of the intestinal flora of these animals for presence and predominance of the organisms isolated from the grains of rice.

It is an admitted fact that extensive discussions and controversies have been carried on for long with regard to the causation of beri-beri. Different varieties of causes have been assigned to this disease, differing widely in nature, but no particular theory as regard its cause, so far as is known, has been definitely accepted, but without fear of contradiction it may be stated that, this disease is most common amongst the rice-eating people, and not confined entirely to the poorer classes, though admittedly they seem to suffer most, living, as they do, under most unwholesome conditions.



My investigation has been restricted mainly to the question whether beri-beri is an infectious disease due to some specific micro-organism, or its toxin, and conveyed directly through the consumption of infected rice

#### NOTES ON SAMPLES OF RICE EXPERIMENTED ON

Some of the samples of rice obtained for the experimentations were from Mandalay, the consumption of which, in a few instances, was definitely associated with beri-beri in man. Through Civil Surgeons in other districts in Burma, samples of rice, reported to have produced the disease in a number of people, were also obtained. Twenty samples (associated with beri-beri cases) thus obtained from the undernoted stations were particularly studied with regard to the physical characteristics of the grains.

Cultural and feeding experiments were also carried out with these samples —

1	Mandalay	4	Akyab
2	Tavoy	5	Mergui
3	Bassein	6	Toungoo

It may be pointed out at the outset that one great disadvantage which was experienced throughout this investigation, and it was no small handicap, was the entire absence of local beri-beri cases, making it impossible to obtain much first hand information on various important points. My personal observations on beri-beri cases during this work had to be confined to those seen, mostly, at the Civil Hospital, Mandalay, during my several visits to that place in this connection. A limited supply of materials was also obtained through the help of some of the private medical practitioners in Mandalay.

Every endeavour was made to get samples of rice actually eaten by the patients before the onset of symptoms, but there was again considerable difficulty in getting these grains, as, by the time the disease started, the bulk of the rice had already been consumed, hence it was difficult to get a sufficient quantity to carry out the experimental feeding of pigeons. However, out of the 20 samples which were studied, 13 were known to have produced beri-beri amongst the consumers and 7 were alleged to have caused the disease amongst labourers in plantations, forests and elsewhere.

The first study undertaken on these various samples was to classify them according to their physical characteristics, adopting somewhat similar methods to those employed by Acton and Chopra (1925) at the School of Tropical Medicine, Calcutta, namely, the water-test.

When studying these it is really surprising to notice what different shades and varieties of rice exist, it is also far more surprising to know what different sorts of grains, as regards their shape, size, colour, etc., are sometimes to be found (in the same sample) mixed up and placed in the open market for sale, the value of each stock depending on the excellence or otherwise of the grains which compose it, ranging from Rs 3 to Rs 7-8 per basket of 20 viss.

It is found that from the time paddy is cropped it takes, as a rule, 6 months to one year before the rice itself, in varying grades, is brought up by rice vendors for sale in the open markets in Burma. Further, it is gathered from rice merchants, that people generally prefer the previous years' stock, as by keeping, it cooks well. Perhaps it may be that the merchants themselves are responsible for this prevalent idea, so that they may be able to clear up their old stocks before touching the new. During this period of delay before consumption, the rice undergoes various vicissitudes of fortune as regards steaming, milling, polishing, transport and storage.

It is pointed out that so far as my observations go, from the 20 samples referred to almost all grades and varieties of rice which are stocked for sale contain the so-called diseased or deteriorated grains, no doubt varying with the quality of the rice. Some appear mouldy, even containing weevils which, it is believed, arise entirely from too much moisture in the rice due greatly to inevitable exposure to rains during transport, and also to faulty and insanitary methods of storage in ill-ventilated places. Such faulty methods of storage lead to deterioration of rice in all possible ways especially if the grains are broken or otherwise damaged in the process of machine milling. One of the chief ways in which such deterioration takes place seems to be the invasion of the interior of the grains particularly the broken and injured grains, by infecting micro-organisms.

Though it is possible, on close scrutiny, with the aid of a magnifying lens, to detect diseased grains, the simple water-test method advocated by Acton and Chopra greatly facilitates such discovery as found by practical experience. This method was invariably adhered to in the picking out of diseased grains from the various samples.

The test consists of putting a small quantity of rice in a dish of water, which brings into great prominence all the various characters of the individual grains. As the authors state, if the grain is allowed to stand too long in the water the fruit becomes swollen and opaque, but this takes some ten minutes or more, so that there is ample time to study the rice. (Acton and Chopra, 1925)

By this method the size, shape and external peculiarities as regards colour, smoothness or coarseness, and the diseased appearance in the interior of the grain, and many other physical defects can be detected.

Except for the fact that the good rice, the so-called Class I rice, is fairly long, thin, smooth and more or less transparent, there is nothing else that is characteristic of it. However, even amongst the best class of rice, shorter and stouter grains are found. The inferior class of rice is distinctly coarser in appearance and much discoloured. The chief characteristic of the diseased grains is the distinct brownish, sometimes even blackish, appearance in the interior of the grain either in its centre, on its sides, or at its ends, usually in the centre. Infection with micro-organisms gains access through the surface of the grains, generally through the embryonic site. It is observed that broken grains are easily liable to be infected with micro-organisms. Badly stored rice has an unpleasant odour. Although opaque rice is universal, some grains appear to be very opaque.

throughout, these are usually found to be diseased, some are waxy in appearance

On examination of the physical characteristics of all the samples of rice which were taken for investigation it was found that they were, judged from the above standards, definitely of inferior quality, not all of them being highly polished

TABLE I

*Showing physical characters, etc., of the 20 samples of rice experimented on*

Number	Station	Physical characters	Particulars
1	Mergui	Polished, coarse stumpy,	Definite number of associated cases not known, but reported to have been the cause of beri-beri among labourers on a rubber estate
2	"	opaque Contained many	
3	"	damaged, discoloured and	
4	"	diseased grains All mouldy	
5	"	and poorly polished except	
6	"	No 1 which was well polished	
7	Akyab	Highly polished appear to be of old stock Coarse, stumpy, broad grains, contained many damaged and diseased grains	Reported to have been associated with beri-beri amongst a large number of Municipal coolies
8	Tavoy	Well polished Short thick grains, contained large number of broken, but hardly any diseased looking grains	Local name 'NGA SEIN' cultivated in Tavoy District Stored in godowns for four months after milling, caused beri-beri in a large group of coolies
9	Bassein	Well polished, thick medium sized grains Contained large numbers of damaged and diseased grains, appeared to be old stock	Definite number of cases associated with this rice not known
10	"	Poorly polished, coarse, broad, stumpy grains a good number being damaged and diseased	Definite number of cases not known
11	Toungoo	A mixture of different grades of coarse grains of various sizes and shapes Badly polished contained many damaged and diseased grains	Local name 'KAUKNGE' Associated with a widespread epidemic of beri-beri among coolies employed on forest operations These cereals were grown in Toungoo District of Upper Burma, and milled at Toungoo The supplies of rice reached the stores in the forest, for the year's supply, during April and May 1927, and was issued in mid-June Stored in original gunny-bags, bags were sprinkled over with lime These two samples got slightly wet, having been caught in a shower of rain just before reaching destination
12	"	Very mouldy, weevils present A very poor quality of rice, badly polished, coarse grains	

TABLE I—*contd*

Number	Station	Physical characters	Particulars
13	Mandalay	Poorly polished, contained fair amount of damaged grains which were stumpy and thick	This was the ration rice obtained from the house of a bandsman of 120th Burma Rifles, whose wife had developed beri-beri
14	,	Well polished thin, medium sized grains contained a fair amount of damaged grains	Local name 'YAZAN' This sample was also obtained from the above-mentioned house Purchased from Mandalay Bazaar, the whole family used to eat it alternately with the ration rice
15	,	Well polished medium sized grains contained a very few broken grains a good clean rice	Local name 'MUDIYA' Associated with a case of beri-beri—a Bengalee doctor practising in Mandalay
16	"	Fairly polished stumpy broad grains contained a fair number of broken grains	Local name 'NAZAMGALE' Associated with beri-beri in a Mahommedan coolie
17	"	Lightly polished, medium sized. Contained many discoloured and damaged grains	Local name 'KALA-O-ZEGWE' Consumed by an Indian cook
18	,	Fairly well polished coarse stumpy grains an inferior looking grade of rice Containing many damaged grains	Associated with a severe epidemic of beri-beri (both dry and wet varieties), amongst the Burmese boarders of the Anglo-Vernacular Training School at Mandalay
19	"	Well polished, whitish looking grains contained a large number of damaged grains	Local name 'MOULOO' Associated with a case of epidemic dropsy in a pregnant Burmese woman who was reported to have aborted after the onset of the disease
20	"	Well polished medium sized, contained a few damaged grains	Associated with a case of beri-beri—a Mahommedan cook

All the samples experimented upon were of the milled variety, some well polished, but a large number less so, and all more or less damaged by milling, so that it would appear that the over polished condition is no criterion that rice for this reason alone would, when consumed, tend to produce the onset of the symptoms of beri-beri. On the other hand, it would appear that the damage caused to the rice while undergoing the process of milling is the chief contributory factor in the deterioration of rice from almost all causes, especially when such grains are exposed to the deleterious influences of climatic and faulty storage conditions. During wet and humid weather such kinds of rice get mouldy and are easily infected with micro-organisms in the manner already described.

## ISOLATION AND CULTURE OF ORGANISMS FROM RICE GRAINS

The technique employed by Acton and Chopra (1925) was adopted for the isolation of organisms from diseased grains

In the first place diseased looking grains were picked up after performing a water-test with a small portion of the sample of rice under investigation. These grains were soaked for half an hour in pure carbolic acid, then washed free of carbolic in two changes of absolute alcohol and finally washed quickly in two changes of sterile water. By this means the grains were well sterilized. They were then fractioned off by cutting them through transversely with a sterile knife, and then planted with the cut end downwards on plates of agar, and incubated under both aerobic and anaerobic conditions. Buchner's tubes with pyrogalllic acid and caustic soda were used for anaerobic method of cultivation. An agar plate devised for anaerobic cultivation by means of pyrogalllic acid and caustic soda method was also used.

TABLE II

*Showing the morphological and biochemical properties and other characters of the three types of micro-organisms isolated*

Character of growth on Agar	TYPE I	TYPE II	TYPE III
	Dry wrinkled, whitish growth tends to spread	Moist brown opaque growth	Moist brown
Morphology	Long bacillus c rounded ends	Slender bacillus c rounded ends	Slender bacillus c rounded ends
Motility	Neg	Neg	Neg
Gram staining	++	+	+
Spore	+	+	+
		Centrally situated	
Liquefaction of gelatin	++	+	+
Glucose	A	A	A
Lactose	Neg	Neg	Neg
Mannite	A	Neg	A
Dulcitate	Neg	Neg	A
Saccharose	A	A	A
'k	A	A	A
		No clot	

A = Acid Neg = No change

It is to be pointed out that not infrequently more than one type of these micro-organisms were isolated from diseased grains picked up from the same sample. Type I was most predominant, then Type II, and Type III was met with only in one sample of rice obtained from Mandalay.

This tends to show that injured grains of rice when exposed to deleterious influences are liable to be attacked by a variety of micro-organisms differing in some points of character, but more or less belonging to allied, though different groups.

From some of the samples of rice aerobic and spore-bearing bacilli, not differing from the Type I organism already described as found in the majority of the samples, were isolated after a portion of the rice had been treated and brought to boiling point for a minute, thus showing that the spore-bearing quality of these bacilli gives them a fair degree of resistance to heat. Attempts were made to cut sections of the diseased looking grains to find out if any micro-organisms were present in the interior of the grains. From a few such sections of different samples, degenerative changes in the interior of the grains were noted along with the presence of a few spore-bearing bacilli.

The technique employed was as follows —

The picked grains were well heated, but not boiled, in a test tube, and then transferred to three consecutive changes of 50, 60 and 80 per cent alcohol respectively.

By this means the grains became sufficiently soft for cutting, afterwards they were transferred to a paraffin bath for two hours and then cut. Long bacilli, some of them with spores, were encountered in these sections, on examination under an oil immersion lens, taking the Gram's stain well. Glassy and transparent looking areas were noticeable in parts of the sections surrounding these bacilli. In some instances the whole field appeared to be hyaline in character, with the bacilli lying in isolated groups. These hyaline areas appear to be areas of liquefaction and other degenerative changes caused by the biological activity of the bacteria living in them.

#### DETERMINATION OF THE TOXICITY, OR OTHERWISE, OF ORGANISMS ISOLATED

The only experiment which was attempted to determine this, was to inoculate rabbits and pigeons with emulsions of 'live' organisms.

Six healthy, medium sized rabbits were used for a total of five subcutaneous injections with six different micro-organisms isolated from the 'beri-beri' rice, beginning with 2 c.c. doses, increasing by 1 c.c. at 6-day intervals, and finishing up with 6 c.c. for the last dose. No signs or symptoms of any disease or discomfort were noticed in any of the rabbits throughout this period, except in two rabbits, where a slight rise in temperature from 100.4°C to 101°C in rectum on the first two injections occurred, subsequent injections did not even produce this sign.

In healthy pigeons the angle was narrower as shown below —



TABLE III

Showing details of each group of pigeons fed on 'beri-beri' rice

Serial Number	Group Number	Sample Number	Days under experiment	WEIGHT IN GRAMMES		Signs and symptoms noted during course of feeding
				Initial	Final	
1	1	11	40	320	192	Green diarrhoea, paralytic gait, wings droop'd, neck stiff and retracted
2			49	352	160	Do
3			62	384	224	Do
4			62	320	190	As above,—retraction of neck only slight
5			46	312	184	As for No 1
6			37	328	202	Do
7	2	12	20	285	224	Green diarrhoea, drooping wings, and staggering gait
8			39	256	160	Green diarrhoea, head slightly retracted, both wings completely paralysed
9			44	416	190	Green diarrhoea, legs paralysed, head retracted, tail propped up
10			55	384	160	Green diarrhoea, drowsy, wings drooped, profound asthenia
11			56	388	178	Green diarrhoea, stiff and retracted neck, legs paralysed
12			60	288	210	Do
13	3	2	25	320	288	Green diarrhoea, marked retraction and stiffness of neck
14			55	266	192	Green diarrhoea, slight stiffness and retraction of neck, blind five days before death
15			65	262	224	Green diarrhoea, high stepping paralytic gait, complete paralysis of legs
16			75	300	224	Green diarrhoea and emaciation
17			65	266	199	Green diarrhoea, stiffness and retraction of neck, paralytic gait
18			42	308	245	Do
19	4	14	18	320	256	Green diarrhoea and marked emaciation
20			31	320	260	Green diarrhoea, slight drowsiness and sudden death
21			32	325	288	Green diarrhoea, drowsiness, drooping, high stepping gait
22			33	256	192	Green diarrhoea and emaciation
23			48	276	224	Green diarrhoea, drooping wings, and staggering gait

TABLE III—*contd*

Serial Number	Group Number	Sample Number	Days under experiment	WEIGHT IN GRAMMES		Signs and symptoms noted during course of feeding
				Initial	Final	
24	5	15	37	256	176	As above,—together with stiffness of neck
25			32	192	141	Green diarrhoea, neck retracted and stiff, wings drooped, paralytic gait
26			16	264	198	Do
27			28	184	256	Do
28			27	320	256	Do
29	6	19	10	426	347	Green diarrhoea, drooping wings high stepping paralytic gait, stiff neck
30			28	252	168	Green diarrhoea, drooping wings
31			22	352	268	Green diarrhoea
32			22	352	288	Do
33			26	320	204	Do drowsy
34			48	336	218	Green diarrhoea, drooping wings, paralytic gait
35			33	384	237	Do
36			31	384	222	Green diarrhoea, retracted neck, legs and wings slightly paralysed
37			37	268	202	Green diarrhoea, legs paralysed, wings drooped
38			52	262	194	Green diarrhoea, paralytic gait, stiff and retracted neck, wings drooped
39	7	18	48	266	198	Do
40			39	306	214	Do
41			30	317	235	Green diarrhoea, drooping wings, high stepping paralytic gait
42			34	284	190	Do

Careful post-mortem examinations of all pigeons were carried out

The technique of dissecting the various organs was learnt from Major C de C Martin, *IMS*, of the Pasteur Institute, Rangoon, and was somewhat on the same lines as followed by Lieutenant-Colonel R McCarrison, *IMS*

The organs, heart, liver, spleen, thyroid, kidney, pancreas, suprarenals and ovary or testes, were removed and carefully weighed

Practically all organs, with the exception of suprarenals, showed distinct signs of atrophy. Spleen, pancreas and ovary showed the greatest amount of shrinkage, heart muscle was somewhat flabby, and showed only a slight degree of atrophy. There was no pericardial effusion. The pancreas was found generally atrophied, sometimes to the extent of weighing only 0.1 and 0.2 gramme, as against 0.94 gramme usually met with in normal state. In many cases



shrinkage of organs was more than half normal weight Kidney showed comparatively the least variation from the normal Lungs were found to be healthy in all the pigeons dissected

Several samples of blood from the hearts of dead pigeons, taken, under sterile conditions, immediately after death and submitted to cultural methods in nutrient broth and on plain agar, proved to be sterile No organisms were found in stained smears of blood from heart, liver and spleen

TABLE IV

*Showing post-mortem findings on pigeons fed on 'beri-beri' rice*

Group Number	Pigeon Number	WEIGHT OF ORGANS, IN GRAMMES						
		Heart	Liver	Spleen	Pancreas	Supra-renals	Thyroid	Kidney
1	1	2.8	5.3	0.05	0.4	0.04	0.02	1.41
	2	2.7	5.3	0.102	0.38	0.01	0.01	1.2
	3	2.1	5.6	0.07	0.7	0.01	0.01	2.1
	4	4.1	4.8	0.12	0.6	0.01	0.01	1.43
	5	3.6	4.9	0.1	0.44	0.019	0.01	1.35
	6	3.82	5.6	0.16	0.68	0.12	0.02	2.0
2	7	2.6	4.9	0.09	0.44	0.019	0.01	1.35
	8	2.4	4.5	0.1	0.1	0.1	0.01	2.0
	9	3.7	5.2	0.1	0.5	0.05	0.07	1.3
	10	3.4	4.8	0.15	0.4	0.015	0.01	1.6
	11	3.1	7.2	0.105	0.58	0.013	0.01	1.6
	12	2.7	5.0	0.08	0.56	0.01	0.01	1.4
3	13	2.7	6.1	0.15	0.4	0.05	0.01	1.8
	14	2.2	3.8	0.1	0.6	0.1	0.01	1.4
	15	2.6	7.5	0.1	0.3	0.2	0.02	1.8
	16	2.6	10.4	0.6	0.2		0.01	1.8
	17							
	18							
	19	2.3	12.5	0.407	0.9	0.01	0.02	2.3
	20	3.1	10.2	0.8	0.5	0.2	0.02	5.4
	21	3.0	10.5	1.2	0.8	0.2	0.02	4.9
	22	3.2	8.6	0.3	0.4		0.02	1.7

TABLE IV—*contd*

Group Number	Pigeon Number	WEIGHT OF ORGANS, IN GRAMMES						
		Heart	Liver	Spleen	Pancreas	Suprarenals	Thyroid	Kidney
4	23	2.1	8.8	0.1	0.4	0.2	0.02	2.2
	24	2.2	8.6	0.2	0.5	0.11	0.01	1.8
5	25	2.85	7.57	0.21	1.02	0.001	0.005	3.63
	26							
	27	2.61	5.46	0.16	0.38	0.15	0.05	1.75
	28	3.8	1.3	0.12	0.1	0.01	0.01	1.5
	29	1.0	7.1	0.08	0.16	0.02	0.02	2.2
	30							
6	31	1.7	5.0	0.05	0.68	0.02	0.07	1.48
	32	3.7	6.3	0.12	0.72	0.03	0.05	1.8
	33							
	34	5.36	9.2	0.14	0.66	0.036	0.01	1.8
	35							
	36							
7	37	3.32	7.18	0.05	0.65	0.05	0.02	2.1
	38	2.8	6.0	0.048	0.63	0.02	0.01	1.8
	39	3.55	5.9	0.11	0.96	0.02	0.01	1.56
	40	3.06	6.85	0.07	0.7	0.01	0.019	3.46
	41	3.3	5.3	0.07	0.65	0.06	0.03	2.2
	42	2.8	4.8	0.09	0.48	0.01	0.01	1.56

EXPERIMENT WITH RICE INOCULATED WITH ORGANISMS ISOLATED FROM  
'BERI-BERI' RICE

A quantity of the best, polished, Mudiya rice, was portioned out into four porous earthenware pots, three of these were mixed with three different types of live organisms isolated from 'beri-beri' rice, the organisms were emulsified in sterile water before being mixed with the good rice, the fourth pot was earmarked as a control. The pots were then stored in a somewhat dark, damp and ill-ventilated place after the rice in each pot had been slightly moistened with a small quantity of clean water. The idea was to try and infect the good rice with a view to isolating, after sufficient time had elapsed the same type of organism from the interior of the grains, and also to carry out feeding experiments

on pigeons to see if these artificially infected grains would cause beri-beri amongst them. Storage for six weeks was considered suitable for this purpose. It was thought that this experiment would help to determine the toxicity or non-toxicity of the organisms isolated.

Three batches of six weighed pigeons were freely and entirely fed on the three samples of rice subjected to infection as stated above. A fourth batch of six pigeons were fed on the controlled rice of this series. No other food formed part of their diet.

The following results were obtained —

Four pigeons of one batch and three of another developed symptoms of polyneuritis within the first two weeks, the majority, during the first week. The minimum number of days required for the onset of definite signs was four, and the maximum, twelve. The usual loss of vigour, green diarrhoea, emaciation, stiffness and retraction of neck muscles, drooping of wings, and progressive paralysis of the legs, were the chief signs noted. In the third batch all developed profuse green diarrhoea and died during the second and third weeks (all but one dying during the early part of the second week) with marked emaciation, none of this batch showed paralytic or other nervous symptoms. In the control batch one died on the third day and another on the sixth day, neither showed any outward manifestations of disease except loss of weight, the remaining four survived and looked active and healthy at the end of the fourth week when the experiment was concluded.

Post-mortem examinations on all pigeons were carefully carried out.

Almost all that died after feeding on the artificially infected rice showed marked atrophic changes in all organs as judged from the loss of weight of the organs, both pigeons of the control batch, which died, showed no apparent changes in the organs.

The four samples of rice used in this experiment were distinctly mouldy, even the controlled sample, and had an unpleasant odour.

On doing the water-test the majority of grains of the three artificially infected samples were found to have a definitely diseased appearance, with black or brown spots in the interior or at the sides of the grains. All of the broken pieces of rice appeared to be severely affected.

The control sample, though it also showed diseased looking grains, was not nearly so bad as the other three. From the interior of the picked grains, from all four samples, spore-bearing organisms were isolated, none, however, showed exactly identical biochemical reaction with those originally used for infecting the grains. All were Gram positive, long bacilli, giving a dry white growth on agar and rapidly liquefying gelatin, they produced acid in glucose and saccharose but none in lactose. Although there was a slight difference in the biochemical reaction it would appear that all belong to the same group, similar to the organisms used for this experiment.

Sections of diseased grains showed the presence of this type of organism in their interior.

This experiment proves that micro-organisms usually met with and isolated from diseased rice grains can be used to infect good rice, that injured rice is more liable to invasion, and that rice, stored under unhygienic conditions of moisture and warmth and, left unexposed to the tempering influence of fresh air, is more likely to become invaded with this class of micro-organism, and to deterioration in other ways

If this short period of storage under conditions identical, or analogous to those, not infrequently, met with in general practice, and not altogether avoidable in many cases, led to infection it can easily be imagined how rice becomes infected during long storage in shops and godowns, especially when outbursts of rain have caught and damaged the rice in the course of transport from one place to another

#### EXAMINATION OF THE INCRETA OF PIGEONS FED ON 'BERI-BERI' RICE

Small loopfuls of several samples of excreta of sickly pigeons were examined both microscopically and by cultural methods. For microscopical examination, ordinary staining by Gram's method, and special staining for spores were employed

In practically every film examined, long spore-bearing bacilli were seen

Nutrient agar plates and McConkey's medium were used for isolating organisms from these faeces

In 11 out of 19 samples of excreta taken, as early as seven days and as late as 29 days after commencement of feeding, and planted on plain nutrient agar, these organisms were isolated. *Bacillus pyocyaneus* was isolated in two of these samples, it may be of interest to note that the majority of organisms grown on plates showed Gram positive reaction

A few controlled tests of similar nature were tried with the excreta of healthy pigeons not fed on 'beri-beri' rice, but fed on paddy and good rice. The interesting point about the excreta of healthy pigeons was, not only the marked macroscopical difference compared to that of the green watery excreta of sickly pigeons, but also the distinct predominance of the Gram negative type

Observations made from this experiment were —

- 1 In the excreta of diseased pigeons, spore-bearing bacilli were found in greater abundance
- 2 The source of this type of bacilli was, possibly, the rice the pigeons fed on
- 3 In the excreta of pigeons fed on 'beri-beri' rice the Gram positive type of bacteria abounded, whereas in healthy pigeons the Gram negative type was predominant
- 4 Bile salt in McConkey's medium did not seem to inhibit the growth of these spore-bearing bacilli

#### CONCLUSION

- 1 The mechanical damage caused to the rice in the process of machine milling which, while it polishes the rice causes great damage to the grains, is the

chief contributory factor to their deterioration when exposed to the deleterious influences of climate

2 The highly polished condition of rice is no criterion that it would, by virtue of that condition alone, tend to produce the onset of symptoms of beri-beri among those who consume such rice

3 The more inferior the quality of rice, the more broken and injured grains does it contain. This injury, under conditions of optimum warmth and moisture and long storage, renders the grains easily liable to infection by a class of rice organisms which invade the interior of the grains through external lesions, the presence of which can be demonstrated in microscopical sections of infected grains and by cultural methods

4 These organisms can be demonstrated in the excreta of pigeons fed on infected rice and, as such, they are non-pathogenic to laboratory animals and possibly to human beings

5 An organism or group of organisms as the sole specific cause of beri-beri has not yet been determined, the poison cannot be of the nature of living germs conveyed through food, as rice is one of the articles of diet which is very well cooked

6 The rice organisms living inside the grains probably produce by their biological activities, some poisonous elements which may be thermostable, not affected by cooking. These poisonous elements may be a factor in determining the onset of beri-beri and epidemic dropsy, or, it may be, that these organisms extract some active principles, such as vitamin B, from the rice grains, the lack of which may produce disease symptoms in man and pigeons

7 The presence of these organisms in rice in large numbers, or, in other words, the presence, in great numbers of diseased grains in rice is an index of its inferiority as regards its unfitness for human or animal consumption

8 It would appear that over-polished or highly polished rice, as such, is not the chief factor to be reckoned with in the causation of beri-beri, in so far as the part played by rice in its causation is admitted, but, on the other hand, it is the problem as to how far rice, damaged in the process of machine milling, tends to become deteriorated on account of bacterial invasion when so exposed to moisture and heat as to permit of its being the easy vehicle of disease process, that should claim the further attention of workers in this field

This work was undertaken with the approval of the Director of Medical Services in India during the year 1927-28, at the District Laboratory, Maymyo, and with the assistance of funds kindly placed at my disposal by the Committee of the Research Fund Association

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# A NOTE ON THE KEEPING PROPERTIES OF POLYVALENT DYSENTERY BACTERIOPHAGE

BY

MAJOR C DL C MARTIN, I M S  
*Central Research Institute, Kasauli*

[Received for publication, August 14 1929]

THE question of the keeping properties of bacteriophage is one of great importance, more especially to medical men in the tropics, who often live under conditions where fresh supplies of this substance cannot be obtained easily. With a view to testing these properties, 76 capsules of a polyvalent dysentery bacteriophage were examined, which had been kept under varying conditions of temperature during a period of 20 months.

The bacteriophage used was manufactured at the Pasteur Institute of Burma, Rangoon, on 23rd October, 1927, from antidysenteric strains both Shiga and Flexner originally supplied by Dr d'Herelle and which had been reinforced later by the addition of many local strains bacteriolytic to both bacilli. After manufacture it had been tested for lytic action against several strains of Shiga and Flexner and it was found that suspensions of these organisms were cleared up completely after a few hours incubation at 37°C. The sterility tests were also negative at this time.

In November 1927, the samples were taken to England via Colombo packed in a trunk in the ship's hold. Hot weather was experienced round Colombo and also in the Red Sea. The bacteriophage was not unpacked in England and in December 1928 was brought back to Rangoon also in the ship's hold when hot weather was again encountered in the Red Sea. From January 1929 to the beginning of June it was stored in a box in Calcutta and in June 1929 was sent up to Kasauli by goods train, arriving here at the beginning of July. The latter journey was made at the hottest time of the year when temperatures between 110°—120°F in the shade are common.

It was therefore decided to endeavour to ascertain whether the varying degrees of temperature to which the bacteriophage had been submitted during a period of 20 months had in any way altered its properties and also whether the bacteriophage was still sterile.

The following procedure was adopted —

(1) *To test the sterility of the tube contents*

(a) Examination by naked eye for evidence of turbidity and microscopically for other evidence of bacterial growth

(b) Four drops of bacteriophage were added to a tube of Douglas' broth and the same amount planted on an agar slope and incubated for eight days at 37°C

(2) *To test the potency of the bacteriolytic action against B dysenteriae shiga (Stokes, Oxford) and B dysenteriae flexner (Hiss and Russell, Bombay)*

(a) A sufficiency of growth from a 24 hour culture on nutrient agar of *Shiga (Stokes, Oxford)* was suspended in 10 c c of broth to give a distinct haziness. Into this was placed one drop of bacteriophage

(b) The same procedure was carried out in the case of the Flexner type of organism

(3) *To test the therapeutic action*, the bacteriophage was tried on two cases of dysentery, the diagnosis of which was confirmed culturally and the results noted

The procedure noted under (1) and (2) was carried out with each of the 76 tubes. The tests were examined for a period of eight days and the results noted daily

*Results of the experiment*

TABLE  
*Bacteriolytic Action*

Numbers of tubes	SHIGA SUSPENSION + 1 DROP BACTERIOPHAGE		FLEXNER SUSPENSION + 1 DROP BACTERIOPHAGE	
	Result after 24 hours	Occurrence of secondary growth on— day	Result after 24 hours	Occurrence of secondary growth on— day
6, 9 to 21, 26, 30, 32, 33, 43, 44, 45, 48, 49, 51, 52, 54, 62, 65, 71.	Clear	<i>Nil</i>	Clear	<i>Nil</i>
7	"	2nd	"	7th
8	"	2nd	"	2nd
46	"	5th	"	5th
61	"	5th	"	<i>Nil</i>
4, 5, 22, 24, 35, 42, 56, 60, 64, 73 to 76	"	<i>Nil</i>	"	2nd
36, 47, 55, 59, 63, 66, 67, 69, 70, 72	"	<i>Nil</i>	"	3rd
23, 34, 39, 40, 50, 53, 57, 58	"	<i>Nil</i>	"	4th
68	"	<i>Nil</i>	"	5th
1 2, 3, 25	"	<i>Nil</i>	"	7th
27, 28, 29, 31, 37, 38, 41	"	<i>Nil</i>	"	8th

(1) *Sterility*

- (a) In none of the tubes did naked eye and microscopical examination show any evidence of bacterial growth
- (b) All the tubes were tested for sterility by the cultural procedure described above. In the cultures from a few tubes, sometimes in broth and on a few occasions on the agar slopes, contamination with *staphylococcus* was found. Re-culturing of the same tubes of bacteriophage gave negative results on two retests, so it is very probable that the growths were due to air contaminations occurring when the cultures were made as this was a very dusty season of the year.

On one occasion however a diphtheroid organism was found and on another, a coliform organism giving the sugar reactions of *B. coli communis*. Retest of the bacteriophage from both these tubes gave negative results on two separate occasions. On no occasion was any pathogenic bacterium of the nature of a dysentery bacillus found.

(2) The suspensions of both types of dysentery bacillus were completely cleared at the end of 24 hours by the drop of bacteriophage in all cases, but on several occasions after the primary clearance a secondary growth reappeared. The results of these experiments are summarized in the Table.

(3) *Therapeutic*

The bacteriophage was tested for its therapeutic properties on two cases —

*Case 1*—A D male adult suffering from colic. Temperature 101°F passing frequent stools of pure blood and mucus. Microscopical examination of the stool showed a typical bacillary dysentery exudate and bacteriological examination showed the presence of an organism giving the cultural reactions of *B. dysenteriae flexner*. One c.c. of bacteriophage in two ounces of water was given three times a day for the first two days. Abdominal discomfort disappeared by the end of the first day and on the second day the stools became fecal, though they were still loose. On the third day a further three doses of 1 c.c. each were given and by the fourth day the patient stated that he had completely recovered. This was a severe test in a way as the patient had to go down to the plains on the day the treatment commenced and carried out his duties without rest or special diet.

*Case 2*—B T male adult. History of dysentery during the war. Now showing well marked symptoms suggestive of early sprue, i.e., sore tongue and passing several copious, pale, frothy and offensive stools during the course of the day. Bacteriological examination of the stools showed the presence of an organism giving the reactions of *B. dysenteriae shiga*.

He was given 1 c.c. of bacteriophage in a few ounces of water three times a day for four days. The condition of the tongue improved, the stools became formed and well coloured and were passed twice a day which he states is normal for him. He states that he is better since taking the bacteriophage than he has been for a long time.

*Analysis of the Table*

It will be seen from the Table that after a primary clearing up secondary growths appeared in the Shiga suspensions on four occasions, namely, in tubes 7 and 8 on the second day and tubes 46 and 61 on the fifth day.



The behaviour of the Flexner tubes was variable. In all 46 out of the 76 tubes examined showed secondary growths from the second to the eighth day

2nd day	14		5th day	2
3rd „	10		7th „	5
4th „	8		8th „	7

Another interesting feature was that in sample 7 there was a profuse secondary growth in the Shiga tube on the second day which again completely cleared up on the eighth day and in sample 8 both the Shiga and Flexner tubes showed a profuse secondary growth on the second day but in this case they both cleared up on the third day. I am unable to explain this phenomenon nor am I able to explain the variation in times of the occurrence of secondary growths in the Flexner suspensions.

### CONCLUSIONS

1 Under the conditions of the test this polyvalent dysentery bacteriophage has retained its bacteriolytic action on strains of *B. dysenteriae shiga* (strain Stokes, Oxford) and *B. dysenteriae flexner* (strain Hiss and Russell, Bombay) which were used in the test, for a period of 20 months

2 It has retained its therapeutic properties in the two cases tested

3 It has retained its bacteriolytic and therapeutic properties under varying degrees of temperature and climate

4 In no single case was there any evidence of the presence of an organism of the dysentery group either in the capsules containing the bacteriophage or in the Douglas' broth and nutrient agar tubes used for testing the sterility of the bacteriophage

I am indebted to Captain H. W. Mulligan, I.M.S., and Major D. Pottinger, M.C., M.R.C.P., R.A.M.C., for affording me the opportunity of testing the therapeutic properties of the bacteriophage on the cases mentioned in the text of this note

# THE CHOLESTEROL CONTENT OF THE BLOOD IN FILARIA

BY

LIEUT-COLONEL T C BOYD, FRCSI, DPH FIC IMS,  
*Chemical Examiner to Government Bengal,*

AND

A C ROY, MSc,  
*Biochemical Research Worker under the Indian Research Fund Association*

[Received for publication August 27, 1929]

WHILE engaged on a study of urinary fat in cases of chyluria we had on many occasions to determine the blood cholesterol content and we noted that the figures obtained were above the average, as determined by us previously in a study of the normal cholesterol content of Indian blood. As, however, only a small number of cases were examined, we refrained from making any comment on the subject at the time. Thanks to the co-operation of Dr Sunder Rao we have since been able to extend our observations over a series of fifty cases of this condition. The actual method employed for the estimation was the same as that described in our previous paper, and our results show an average cholesterol content in filaria of 0.146 per cent with a maximum and minimum range of 0.22 per cent and 0.12 per cent. The actual condition present in the patient and the individual cholesterol content are shown in the following Table.

## CONCLUSIONS

- (1) In filaria the blood cholesterol content is increased.
- (2) The maximum noted was 0.220 per cent and the minimum 0.12 per cent with an average of 0.146 per cent as against the normal average of 0.116 per cent.

Our thanks are due to Dr Sunder Rao for having supplied us with material and also to the Indian Research Fund Association for a grant.

TABLE

Number	Age, nationality	Other particulars	Cholesterol content per cent
1	22, Hindu male	Filarial orchitis	0.15
2	40, " "	" lymphangitis	0.133
3	22, " "	" "	0.15
4	20, " "	Microfilaria +	0.133
5	20, " "	Filarial lymphangitis	0.14
6	10, " "	Elephantiasis	0.133
7	17, " "	Filarial lymphangitis	0.12
8	45, " "	Microfilaria +	0.133
9	50, " "	" "	0.12
10	22, " "	Filarial lymphangitis	0.15
11	46, " "	Microfilaria +	0.133
12	20, " "	Filarial lymphangitis	0.12
13	17, " "	Elephantiasis	0.133
14	17, " "	Filarial lymphangitis	0.16
15	30, " "	" "	0.133
16	42, " "	Filarial lymphangitis and elephantiasis	0.12
17	48, " "	Filarial elephantiasis	0.20
18	30, " "	Elephantiasis scrotum	0.13
19	21, " "	Filarial lymphangitis	0.13
20	46, " "	Filarial elephantiasis	0.12
21	48, " "	Filarial lymphangitis	0.15
22	39, " "	Filarial scrotum	0.18
23	22, " "	Filarial lymphangitis	0.12
24	20, " "	Filarial elephantiasis	0.12
25	35, " "	" "	0.16
26	40, " "	Filarial lymphangitis	0.20
27	35, " "	" "	0.12
28	12, " "	" "	0.12
29	22, " "	Elephantiasis and lymphangitis	0.133

TABLE—*contd*

Number	Age, nationality	Other particulars	Cholesterol content per cent
30	27 Hindu male	Filarial lymphangitis	0.15
31	36, , "	" "	0.15
32	40, , "	Elephantiasis	0.16
33	45, , "	Filarial lymphangitis and abscess scrotum	0.17
34	40, , "	Chyluria	0.17
35	50, , "	Lymphangitis scrotum	0.17
36	23, , "	Elephantiasis	0.12
37	52, , "	Filarial lymphangitis	0.15
38	20 , , "	"	0.16
39	45 Mohammedan male	Microfilaria +	0.15
40	52, , "	"	0.14
41	35, , "	"	0.14
42	22, , "	Elephantiasis	0.12
43	25, , "	Filarial lymphangitis	0.12
44	30, , "	Filarial lymphangitis scrotum	0.20
45	35, , "	Filarial scrotum	0.17
46	22, , "	Elephantiasis	0.12
47	35, , "	Filarial orchitis	0.22
48	35, , "	Filarial scrotum	0.16
49	42, , "	Elephantiasis	0.16
50	36, , "	Elephantiasis scrotum	0.16

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A NOTE ON TWO SPECIES OF INDIAN ANOPHELINE  
MOSQUITOES—*A. INSULÆ FLORUM* SWELLENGREBEL  
AND *A. AITKENII* JAMES, WITH ITS VARIETY  
*BENGALENSIS* NOV. VAR.

BY

I. M. PURI, M.Sc. (Punjab), Ph.D. (Cantab.),

*In-charge of the Inquiry on the Larval Characters of the Anopheline Mosquitoes  
of India*  
(Central Research Institute, Kasauli)

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SWELLENGREBEL (1919) has described, from Dutch East Indies, a number of different forms of larvæ belonging to the species *A. aitkeni* James, most of them differing from one another mainly in the branching of the inner anterior clypeal hairs. Judging from the collections of Anopheline larvæ made so far from various parts of India, it appears that larvæ of only three of these forms occur in this country—firstly those with the inner anterior clypeal hairs simple, secondly, with the inner anterior clypeals splitting into two a little beyond the base, and thirdly, with the inner anterior clypeals splitting into 3 to 5 branches in the distal half.

Among the Indian Anopheline mosquitoes *A. aitkeni* is the only species the larvæ of which seem to possess such marked and constant differences in certain characters without any intermediate forms and it is of interest to know what specific or varietal value these differences have and also whether the adults of these various forms show any corresponding differences from one another.

The larvæ with the simple inner anterior clypeal hairs agree in all essentials to Swellengrebel's (1920) description of *A. aitkeni* var. *insulæ florum*. A close study of these has, however, shown that, as already pointed out by Christophers (1924), the larval characters are so different from those of *A. aitkeni* that they denote a distinct species. Moreover, a detailed examination of the adults, reared from such larvæ has revealed that the genital armature of the male also has certain well marked differences from those of *A. aitkeni*. In view of the presence of such distinct and constant differences in certain characters of the adults as well as of the larvæ, there seems no doubt that *insulæ florum* is a true species distinct from *A. aitkeni* James.

Of the other two types of larvæ, the one with the inner anterior clypeal hairs split into two has so far been found in India in the South only, from where *A atkenu* was originally described, and obviously belongs to the type form of this species. Larvæ with the inner anterior clypeal having 3 to 5 branches have so far been collected from India in the North-East only.

These two forms of larvæ exist quite distinct from one another without any intermediate condition and occur in separate localities, so far as India is concerned, and although the females reared from them are absolutely alike, the genital armature of the males show certain differences from one another in their detailed structure. In view of these facts it seems advisable to distinguish the form occurring only in North-East India, as a separate variety distinct from the type form of *A atkenu* James. On account of the restricted distribution of this variety to North-East India only, I have called it *bengalensis* nov. var. of *A atkenu*.

The adults of these three forms resemble one another very closely, differing only in the structure of the male genitalia, which have been described for the first time below. Detailed descriptions of the larvæ of these forms will be given along with those of the other Indian Anophelines and some of the important characters only have therefore been dealt with here.

#### *A insulæ florum* Swellengrebel (1920)

*Adult*—Genital armature of the male.—*Ninth segment* narrow, without ventral processes. *Side pieces*—Parabasal spines 2, outer much longer than the inner, arising from slight prominences, ends drawn out and recurved. A number of well-developed hairs cover the dorsal and most of the ventral surface. Accessory hairs absent. A well-developed internal spine arises near the distal end of the side piece. Harpogones of the two sides fused in the middle line, each is distinctly three lobed. The ventral lobe has three stout sword-like chætæ which appear to be partly fused in the basal one-third. The middle lobe has two sword-like chætæ arising close together. They are slightly longer than those on the ventral lobe and are expanded distally. The dorsal lobe has three long chætæ arising separately, one of them arising at the apex has a short accessory hair slightly external and ventral to it. A number of non-papillated hairs present on the inner portion of the harpogones near the middle line. All the chætæ are comparatively longer than those in *A atkenu*.

*Edæagus* poorly chitinized, broad papilliform, with a few short subapical papilliform leaflets directed backwards. No terminal thickening.

*Anal lobe* broad, reaching half-way down the side pieces. Harpal thickening not apparent.

*Larva*—Anterior clypeal hairs simple, inner anteriors arising close together, posterior clypeal hairs split near base into three or four branches. Palmate hairs developed on metathorax and on abdominal segments I to VII. Balancer or lateral hair on the third abdominal segment only half as stout as on segments

I and II, and has only a few branches (5 to 8) Lateral or the saddle hair of the anal segment often simple

*Distribution*—I have collected larvæ of this species from Marianbarie (Bengal Terai, March 1928), Sukna (Darjeeling District, September 1928), and Yellapur (Bombay Presidency, February 1927) Cogill (1903) has described them from Karwar and Carter (1925) from Ceylon

*A. atkensis* James (1903)

*Adult*—A complete description of the genital armature of the male has already been given by Christophers (1915), but for the sake of comparison with the genitalia of the proceeding species the harpagone and the œdæagus have been redescribed below —

*Harpagones* fused in the middle line, trilobed Ventral lobe has three sword-like chætæ arising close together and may be fused partially in the proximal one-third The middle lobe has two sword-like chætæ arising close together, slightly longer than those on the ventral lobe and expanded distally The dorsal lobe has two stout chætæ arising separately and a third, smaller than the other two, arising near the apex with a very short accessory hair lying ventral and a little anterior to it A number of minute non-papillated hairs on the inner portion of the harpagones near the middle line

*Edæagus* poorly chitinized, papilliform and without leaflets or terminal thickening

*Larva*—Each inner anterior clypeal hair splits into two branches at about one-fourth of their length from base Distance between the bases of the two hairs is about equal to that between the bases of the inner and the outer anterior hairs of one side Outer anterior clypeal hairs split into 2 to 5 branches in the distal one-third, posterior clypeals divide near the base into 3 to 7 branches Palmate hairs developed on metathorax and on abdominal segments II to VII Balancer or lateral hair of abdominal segment III like those on segments I and II Saddle hair of the anal segment has 4 to 5 branches \*

*Distribution*—Larvæ of this species have been collected by me from Yellapur, Ramangali (both in Bombay Presidency, February 1927), and Coonoor (Nilgiri Hills, December 1927), and from Mercara (Coorg, by Dr K R Rao, October 1928) and have been described from Karwar by Cogill (1903) and from Ceylon by Carter (1925)

*A. atkensis* var *bengalensis* nov var

*Adult*—The genital armature of the male of this variety resembles that of the type species except that there are only two sword-like chætæ on the ventral



lobe of the harpagones instead of three as found in the latter. Moreover, the various chaetae arising from the lobes of the harpagones are comparatively shorter.

*Larva*—It differs from that of the type species only in the branching of the inner anterior clypeal hairs. In this variety the inner anterior clypeal hairs split into 3 to 5 branches about one-third the length of the hair from its base.

*Distribution*—Larvæ of this variety have been collected so far from Marianbarie (Bengal Terai) February and September 1928, and from Sukna, September 1928. Probably most of the records of the presence of *A. aitkeni* in Bengal and Assam are of this variety, only a few being of *A. insulæ florum* which is not a very common mosquito.

*Type*—Type male and female, reared from isolated larvæ with the genital armature of the male mounted in balsam on a slide, are in the British Museum (Natural History). Paratypes, also reared from isolated larvæ and with the male genital armature mounted in balsam, are in the Indian Museum, Calcutta, and in the collection of the Malaria Survey of India, Kasauli.

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# THE WASSERMANN REACTION IN KALA-AZAR

BY

LIEUT-COLONEL R B LLOYD, M A , M B , B Chir , I M S ,

*Imperial Serologist,*

L FEVERARD NAPIER, M R C S , L R C P ,

*In-charge Kala-azar Research Department Calcutta School of Tropical Medicine  
and Hygiene,*

AND

RAJ G C MITRA, BAHADUR,

*Assistant Serologist*

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## INTRODUCTORY

As kala-azar is a chronic disease, associated in its fully developed phase with a high globulin content of the serum, it seemed important to examine the Wassermann reaction in a large series of cases of kala-azar in order to obtain further information on the question whether infection with *Leishmania donovani* causes a positive Wassermann reaction, or alternatively, if syphilis is an aetiological factor of any importance in the genesis of kala-azar

## LITERATURE

Kala-azar being a disease of comparatively limited distribution, the literature contains but few references to this question

Sutherland and Mitra (1915) in Calcutta examined the Wassermann reaction in 38 definitely diagnosed cases of kala-azar, in all of which leishmania had been demonstrated. They found ten positive reactions, but they added that 'of the ten positive cases only two gave a more than slightly positive reaction'. Being intimately acquainted with the Wassermann technique by which these results were obtained, we are in a position to say that the eight slightly positive reactions would now be classified as incomplete negative reactions. Thus only two definitely positive reactions were obtained in 38 cases, a result which can be quite adequately accounted for by associated latent syphilis

Iyengar (1923) examined the Wassermann reaction of 30 definitely diagnosed cases of kala-azar, all under treatment, in all of which leishmania had been demonstrated. The Wassermann technique used was the well-known Medical Research Committee (now Council) method No. 4. Six positive reactions were obtained (20 per cent). Iyengar refers to his previous study of the Wassermann reaction in an unselected apparently healthy Indian male population of 400 persons in which he obtained 88 positive reactions (22 per cent), and concludes accordingly that kala-azar infection is not a cause of a positive Wassermann reaction.

Megaw and Mullick (1928), in a paper analysing the results of laboratory findings obtained by the research departments of the Calcutta School of Tropical Medicine and Hygiene from patients of the hospital attached to that school, report the Wassermann reactions in 400 unselected cases, of which 90 were kala-azar. These Wassermann tests were performed in the laboratory of the senior writer.

The 90 kala-azar cases showed 6 strongly positive reactions and 25 moderately positive, or a total of 31 positives (34.4 per cent).

The 310 non-kala-azar cases showed 19 strongly positive and 51 moderately positive reactions, i.e., a total of 70 positives (22 per cent). This latter figure is identical with Iyengar's figure for an unselected Indian male population, and approximates very closely to the estimated syphilis rate of the Calcutta hospital population which is believed from evidence of various kinds to be around 20 per cent.

A series of 90 cases is not of course a very large one from which to calculate percentages, but as far as it goes, it shows a definitely higher percentage of positive Wassermann reactions in kala-azar than in the controls, though the percentage is not sufficiently high to encourage the view that kala-azar is a cause of a positive Wassermann reaction.

Megaw and Mullick state that —

'The high proportion of positive findings in cases of kala-azar is capable of two interpretations —

(1) That the blood changes in the disease accentuate the serological conditions which are responsible for the positive Wassermann findings, or (2) that the presence of the syphilitic virus predisposes to the occurrence of kala-azar. There are grounds for believing that lowered resistance is an important factor in predisposing to kala-azar, and it is quite possible that syphilis may be one of the causes of the lowering of resistance to the disease.'

#### OUR OWN SERIES

Complete records being available of a very large number of kala-azar cases which have been under the care of one of us (L. E. N.), we are able here to present the large series of 474 cases in which the Wassermann reaction has been examined. Every case included in the series was a definitely diagnosed case in which leishmania had been demonstrated. Cases in regard to which there was any doubt as to the diagnosis were excluded. The series was, otherwise, entirely

unselected. The Wassermann tests were carried out in the laboratory of the senior writer by a method, previously published, which has been in use many years and which is known to be absolutely satisfactory.

The results obtained were as follows —

Strongly positive	30		
Moderately positive	75	Total positive	105
Negative	306		
Incomplete negative	63	Total negative	369
	<hr/>		<hr/>
	474		474

The percentage of positive reactions is thus 105 in 474 or 22 per cent. This rate is no higher than the estimated syphilis rate of the controls. We conclude, accordingly, that kala-azar infection is not a cause of a positive Wassermann reaction. It is necessary to state here that Megaw and Mullick's series of 90 cases, which showed a distinctly higher Wassermann-positive percentage than the controls, were all examined in the senior writer's laboratory, and are all included in our own large series of 474 cases.

The senior writer also examined in 1921 the Wassermann reactions in another series of 101 definitely diagnosed cases of kala-azar, the presence of leishmania being confirmed in every case. In some cases this was done by one of us (L. E. N.), and in others by Colonel F. P. Mackie, R.M.S., whose help is gratefully acknowledged. This series, the results of which have not been previously published, showed 18 strongly positive reactions and 11 moderately positive reactions, i.e., approximately 29 per cent. This is a somewhat higher figure than the percentage ordinarily yielded by control series.

### CONCLUSIONS

We therefore reach the following conclusions —

- (1) Kala-azar is not a cause of a positive Wassermann reaction.
- (2) Syphilis may possibly have some slight influence in determining an attack of kala-azar, cases of which would in consequence tend to show a somewhat higher proportion of positive Wassermann reactions than the controls. Of this, however, there is but little evidence.

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# AN IMPROVED CITRONELLA MOSQUITO DETERRENT.

BY

CEDRIC DOVER

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CITRONELLA oil and the various proprietary preparations containing it are of necessity extensively used in the tropics, but their volatility renders their effect very transient, the pure oil being also often irritating to the skin. The use of liquid vaseline, which retards the volatility of the oil, as a base, or the addition of spirits of camphor and cedar-wood oil,\* produces more satisfactory but no means ideal, results, and has suggested to me the formula which follows. It has been used with great success for the last two years in India, Burma and British Malaya.

Citronella oil (Burgoyne's)	1 oz.
Spirits of camphor	1 oz.
Cedar-wood oil	1 oz.
White petroleum jelly (B. P.)	2 oz.

The petroleum jelly should be melted and the other constituents added, the mixture being well-stirred. Bottle (a 3 oz. wide-mouthed jar of suitable size) and cool rapidly, preferably by placing the bottle (which should be closed) in a basin of cold water or in a refrigerator.

The formula gives a firm, whitish, non-staining cream which, in addition to its properties as a mosquito-deterrent, is also soft and beneficial to the skin (petroleum jelly, it will be remembered, is one of the most face creams). One application usually lasts for a whole day, and a very small quantity need be used on each occasion. To soothe the face in the evenings, the cream has been employed by some people to keep the hair, as it was found that for a time this keeps moist and soft as successfully as if the whole face were smeared with it.

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\* Cf. Hungerford, 'Insect Pests about the House' Twenty Years of the Kansas State Board of Agriculture, p. 7.



# TESTS ON THE EFFECTS OF COUMARIN ON THE LIFE OF THE MOSQUITO AND THE MALARIA PARASITE

BY

BRUCE MAYNE,

*Malariologist, Malaria Survey of India*

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## INTRODUCTION

It has been claimed that clover and other leguminous plants have certain relationships to the control of malarial fevers in rendering cultivated sections immune from the disease. The only available references in the literature in this connection may be summed up as follows —

Willcocks (1927)\* attributes the relative immunity of Egypt from malaria to 'something in all leguminous plants, especially in certain kinds of clover which makes mosquitoes immune from malaria'. He quotes d'Herelle stating that in all malaria free regions of Argentina, there is a scented clover *Melilotus altissima* whose blossoms are frequented by the malaria mosquitoes which feed on the syrup coumarin. Willcocks asks, 'May not this act on the mosquito as quinine does on man?' *Melilotus* introduction he states has coincided with expulsion of malaria from certain islands of Zealand and from the northern provinces of Holland.

It has never been intimated how the active chemical substance in clover and other leguminous plants affects the dissemination of malarial fevers. Is it by its action on the insect host or on the parasite it carries? The only suggestion in this regard is that made by Willcocks (1927) who indicates that 'it may act on the mosquito as quinine does on man'.

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\* Willcocks, William (1927) 'Why is cultivated Egypt immune from malaria,' Nile Mission Press, Cairo, Dec 1927, 13 pp and Discussion. Reviewed in *Trop Dis Bull*, 1928 Vol 25, No 7, July, p 557 (by Lane, C)

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The substance contained in legumes which is reputed by all writers in this connection to be effective is coumarin. It was not possible to obtain in India a natural coumarin syrup for purposes of this investigation so recourse was had to the best quality of coumarin of commerce obtainable.

Coumarin crystals used in the tests to be described were secured from a reliable chemist's shop in Calcutta. The chemical was said to contain the anhydride of ortho-oxycinnamic acid ( $C_9H_6O_3$ ) a crystallizable odorous substance, found in several leguminous plants including berseem, Egyptian clover.

The following information concerning coumarin was furnished through Dr F J F Shaw, Imperial Economic Botanist, by Dr Sen, Imperial Agricultural Chemist of the Indian Service.

Coumarin is the active odorous principle present in Tonca Bean *Depteyn odorate* (occurring up to 3 per cent). It is also present in leaves of *Liatris odoratissima* (deer's tongue, hound's tongue). Coumarin has been also found in many other plants, including *Melilotus officinalis*, *M. hamatus*, *M. albus*, *M. leucanthus*, *M. altissimus*, *Trifolium melilotus*, etc. The chemical which is nowadays prepared synthetically, finds a use in perfumery. 'Newmown Hay' is a favourite perfume in which coumarin is the chief ingredient, and the substance is also a normal constituent of lavender oil.

#### PROCEDURE

The crystals were made up into a stock solution of 1 part to 1,000 parts of distilled water and diluted as required for application. It was used by saturating the pads, which lined the wooden trays containing the lamp globes which confined the mosquitoes undergoing test. In addition, small cotton lint pads placed above the netting of the globes were soaked with the material. When raisins were used to furnish fruit juice, these likewise were kept moistened with the diluted coumarin.

It was endeavoured to keep these pads constantly moistened, and at least three times daily they were freshly supplied with liquid.

When the diluted chemical was used for mixing with sporozoites obtained from gland dissections, the material was mixed with normal salt solution.

The following tests were made with captured female Anophelines of the three common species *A. culicifacies*, *A. subpictus* and *A. fuliginosus*. There were no specific differences observable in the effect of the chemical, so the three species are regarded as of equal interest in this connection.

The first attempts were made with the solution given in the various dilutions tabulated, but it was soon realized that the mosquitoes could not be maintained alive without the addition of fruit juice in the form of raisins soaked with the chemical solutions.

Table I gives the details of these tests indicating parallel treatment with the chemical alone and with the addition of fruit juice.

TABLE I

*Effect of exposure of anophelines to various solutions of coumarin*

WITHOUT FRUIT JUICE		Strengths of solution	WITH FRUIT JUICE	
Number of mosquitoes exposed	Maximum length of time mosquitoes survived		Number of mosquitoes exposed	Maximum length of time mosquitoes survived
200	4 hours	1—1 000	50	4 hours
150	12 ,	1—1 500	30	36 ,
25	2 days	1—1 750	50	4 days
25	4	1—2 200	50	4
100	3	1—2 250	85	11 ,
85	3	1—2 750	100	13 ,
50	4 ,	1—3 000	100	14
35	3 ,	1—3 500	35	15 „
45	3	1—3,750	50	20 ,
40	5 ,	1—4,000	45	11 ,
120	4 ,	1—5 000	40	14 „
65	3	1—7,500	50	14 „
75	4	1—10 000	50	15
100	4 ,	1—12 500	145	18 ,
40	4	1—25 000	50	16
75	4 ,	1—50 000	50	15 ,
50	4 ,	1—500,000	50	15 ,
Control with water alone			Control without chemical	
115	4 days		140	19 days

## SUMMARY OF TABLE I

It is evident that the mosquitoes could not live more than 5 days when treated with various strengths of coumarin solution unless fruit juice was provided at the same time. Controls given water alone lived a maximum of 4 days.

Mosquitoes survived the exposure to coumarin solutions of 1—1,000 and 1—1,500 from 4 hours to 36 hours whether they had access to fruit or not.

The greatest length of time any of the anophelines lived was 20 days when having access to coumarin solution of 1—3,750 strength. The controls lived a maximum of 19 days.

It is indicated, from the results of these experiments, that coumarin in solution in the strengths employed did not exert any deleterious influence on the lives of the Anophelines exposed

THE EFFECT OF COUMARIN ON THE MALARIA PARASITES WITHIN THE MOSQUITOES

The mosquitoes employed in this test were specimens of *C fatigans* fed on birds harbouring avian plasmodia. The mosquitoes had been reared in the liquid in which they had been collected as larvæ and mixed with coumarin solution at a strength of 1—20,000. The adults were treated as two batches, with untreated controls of 45 and 65 mosquitoes, respectively. The trials with the solution of 1—1,000 strength were made with infected mosquitoes which had previously undergone an incubation period of seven days. The remainder of the mosquitoes were killed for dissection in the stages of development indicated in the column marked 'Period of application'

TABLE II

*Effect of coumarin on the malaria parasite within the mosquito*

Strength of solution	Number of mosquitoes tested	Period of application	Result of dissections	Dissected controls untreated
1—1,000	20	4 hours	16 with oocysts and active sporozoites	After 10 days 40 out of 45 positive
1—3,500	1	6 days	Typical live parasites	
	5	9 "	4 with typical live parasites	
	19	10 "	12 with numerous active parasites 2 with few parasites	
	45	11 "	42 with typical live sporozoites	
1—7,500	2	5 "	Both quite positive	65 in various stages of parasite development during 15 days
1—10,000	30	8 "	29 with sporozoites actively motile	
1—20,000	2	3 "	Both with oocysts having motile pigment	
1—500,000	20	5 "	19 quite positive	

SUMMARY OF TABLE II

An examination of the results of these tests indicates that there is substantially no lethal effect of coumarin solution in the strengths used on the parasites of malaria in the mosquitoes tested

In 144 specimens tested, the development of the malaria organism was observed in 124 of them. It is interesting to note that although 16 out of 20 specimens involved succumbed when exposed to the action of 1—1,000 coumarin solution, their parasites evidently were not affected.

#### Effect of coumarin on the sporozoites

It was desired to learn the lethal action of the chemical on extracted sporozoites. For this purpose various strengths of coumarin were prepared by mixing with normal saline and used for the dissection as well as for the treatment of the contents of the extracted salivary glands. The effect on the sporozoites was timed at the moment of successful dissection. They were regarded as dead when

TABLE III  
*Effect of testing sporozoites with coumarin*

Strength of solution in saline	Period of infection mosquitoes used	Length of treatment	Effect on sporozoites	Results of bird inoculation
1—5,000	13 days	15 minutes	Killed	Negative
	13 "	15 "	"	"
	13 "	18 "	"	"
	13 "	25 "	"	"
1—10,000	14 "	15 "	"	"
	14 "	25 "	"	"
1—12,500	15 "	20 "	"	"
	15 "	30 "	"	"
1—13,500	10 "	45 "	No effect on motility	Positive
	10 "	1 hour	"	"
	10 "	1½ hours	"	"
	10 "	5 "	"	Not injected
1—15,000	16 "	45 minutes	"	Positive
	16 "	45 "	"	"
	16 "	40 "	"	"
	16 "	40 "	"	"
1—25,000	15 "	30 "	None	"
	15 "	43 "	"	Not injected
1—50,000	15 "	40 "	"	Positive



# ADDICTION TO 'POST' (UNLANCED CAPSULES OF *PAPAVER SOMNIFERUM*) IN INDIA

BY

LILUT-COI R N CHOPRA, MA MD (Cantab), IMS,  
In-charge Drug Addiction Inquiry, Indian Research Fund Association,

IN COLLABORATION WITH

KHEM SINGH GREWAL, MB, BS,  
JOGINDRA SINGH CHOWHAN MB BS,

AND

GU RBUKSH SINGH CHOPRA MB BS  
(From the Department of Pharmacology, Calcutta School of Tropical  
Medicine and Hygiene)

DRUG ADDICTION SERIES No 5

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## HISTORICAL AND GENERAL

THE properties and uses of the capsules of the opium yielding papaver were known long before the Christian era. According to De Candolle, *Papaver somniferum* or opium yielding poppy is a cultivated state of *Papaver setigerum*. Various species of the poppy have been cultivated as ornamental garden plants and have been mentioned by the writers from the earliest times. There is little doubt that the merits of the seeds as a food were recognized much earlier than the somniferous property of the capsules and it is also certain that the soporific and narcotic properties of the capsules themselves were appreciated long before their recognition in its milky sap. The capsules have been employed in the preparation of soporific drugs or in the preparation of stimulating and soothing beverages from times immemorial. According to Watt, *Papaver somniferum* was grown in Asia Minor many centuries ago for its capsules, and the Arabs carried the dried poppy heads to the eastern countries including China even before the inspissated juice was taken and its properties made known to the inhabitants of those regions. The medicinal properties

of the plant and its capsules were fully known during the early classic period of Greece and Rome. One of the earliest references to opium appears to be about the time of Theophrastus who lived in the beginning of the 3rd century B.C. and who seems to have been acquainted with the preparation and uses of the juice of the poppy. There appears to be no doubt that the value of the seeds and capsules was known prior to that. The Egyptians had been using poppy capsules in the 1st century A.D. The early Chinese works mention the Arabs exchanging poppy heads with Chinese merchants. When the capsules were first shown to them, their urn-shape and millet-like seeds suggested the name *Mi-nang* (millet vessel) and *Yingsu* (jar millet). There are records to show that the Arabs instructed the Chinese to prepare from these capsules a soporific beverage and medicine before they knew anything about the properties of opium. There appears to be no doubt that the word *Ya-pien* (opium) followed the *Mi-nang*.

It will thus be seen that capsules of the poppy aroused the attention of the human race long before opium was known. Little wonder then that after their and soothing properties were appreciated by those practising in the East, they became known to the laity who made use of them for purposes of the almost universal desire which human beings possess for a stimulant and sedative.

#### MEDICAL USES OF POPPY CAPSULES

Poppy heads are not commonly used nowadays in medicine but we have seen their employment for medicinal purposes in the early classic Greek and Roman times as well by the Egyptians during the reign of the later dynasties. They have been used in both the Ayurvedic or the Hindu medicine and the the Mohammedan medicine for many centuries as a sedative both for internal use and external application. The '*hakims*' prescribe them for headache, neuralgia, dysentery and digestive troubles in children. They are used as a household remedy in many parts of India and are given during the teething periods by mothers to their children to keep them quiet. An infusion prepared from the poppy heads is used as a soothing application for bruises, inflamed, excoriated and swollen parts and sometimes as an application for various forms of painful conjunctivitis, inflammation of the ears, etc. Fomentations with poppy heads are even now applied to painful inflammatory swellings. Even in China the physicians used them freely in the early centuries of the Christian era. Most of the Lung dynasty medical writers and from them downwards extoll the merits of poppy capsules in the treatment of dysentery, especially when combined with astringent drugs. The Chinese writer Wang-Shih in his work *1-Chien-Fong* says the effect of poppy capsules in dysentery is simply magical. According to Dr. Edkins both the red and white forms of poppy were certainly described and used in the Chinese medicine in the 11th century before opium was known. A medical author of the Yuan dynasty (13th century) describes the preparations of poppy capsules as a very effective remedy against dysentery.

# USE OF POPPY CAPSULES FOR EUPHORIC PURPOSES

It is well known that the use of articles of stimulative restorative or sedative character, is bound up with the natural history of human beings from the very earliest times. The use of such articles as cocoa coffee tea, opium alcohol, etc., to procure an added feeling of pleasure, has been recorded long before the history of civilization. All of them, in moderate quantities produce a favourable effect on the mental conditions of man. Whether they have a stimulating or a depressing effect on the central nervous system, they all produce an enhanced sense of well-being or euphoria. The capsules of the poppy were used very early for this purpose. Whatever may have been the case in the countries of its origin (e.g. Asia Minor) there appears to be little doubt that poppy heads began to be used for euphoric purposes in India soon after the introduction of the poppy plant in the country. There the plant was known as *Koknar* the capsules were called *goza*, *lhol-i-koknar* or *post i koknar* or simply *post* or *post doda*. In the time of the Moghuls a beverage made from the poppy capsules known as *kulnar* was very commonly used throughout the country. Abul-Fizl in his *Ain i akbari* mentions about the Emperor himself taking this drink. He says 'whenever His Majesty is inclined to drink wine or take opium, or *kulnar*, trays of fruit are set before him'. The use of the word *kulnar* apart from opium in the above passage shows that both the poppy capsules and the inspissated juice or *Afyun* were used. According to Watt the beverage *post* at present taken in the Punjab closely resembles *kulnar* which was a luxury among the Mohammedans in the time of Akbar. There is also mention of a beverage known as *Chai-bughra* which was a mixture of wine, hemp opium and poppy capsules. Many other references in the Moghul literature indicate the extent to which the habit of drinking *post* or *kulnar* prevailed among the Indians during the 16th century and later. Bontems, writing of Batavia in 1658, divided the Indians into *Push*, i.e. those addicted to poppy capsules and *Afyun* or those taking opium. During the 17th and 18th centuries the use of *post* was very prevalent as is evident from the remarks of various writers of that period\*. The people in those days grew poppy and used it in any way they liked, the use of the capsules for euphoric purposes appears to have been very prevalent for that reason. In the history of the Punjab during the time of the Sikhs there are many references to *post* drinking, but it is not possible to form an idea as to the extent to which the habit prevailed among the people. Since the introduction of restrictions in the cultivation of the poppy the temptation has been undoubtedly removed from the doors of the peasant and we have no doubt that the habit has considerably decreased for that reason. Poppy heads are obtained now with difficulty and in

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\* The general massacre at Delhi by the Persian invader Nadir Shah, it is said, was due to the ravings and hallucination of a *post* addict. In *Jehan fashai nadari* (a book in Persian) the author writes that an addict under the effect of the drug suddenly said that Nadir Shah was dead. This news was heard by the other addicts and repeated. A soldier named Ahmed Shah heard this, stood up in rebellion and killed a few soldiers of Nadir Shah's force. This enraged the invader king and he ordered a general massacre.



most parts of India the beverage post has to have been replaced by opium. It has been said that the use of poppy capsules or post has become very uncommon in this country.

### CONTROL EXERCISED ON THE PRODUCTION, POSSESSION AND SALE OF POPPY HEADS

Although no statistics are available from the numerous popular stories that are current among the people, it would appear that addiction to poppy heads must have been much more extensive in the Punjab in the past than it is at the present time. In days gone by poppy was grown to a greater or lesser extent throughout the plains of the Punjab. In the valley of the Beas river east of Lahore, it was cultivated up to an altitude of nearly 7,500 feet above the sea-level. Most of the western districts grew poppy for the local use. Poppy cultivation is now mainly confined to the United Provinces under strict Government supervision. The only other parts of British India where poppy is allowed to be cultivated are limited areas in the Punjab.

To trace briefly the history of poppy cultivation in the Punjab under the British régime, the poppy was freely cultivated in the Punjab before the British administration and there was a very wide range of addiction to poppy heads. The Government realized from the very outset the injurious effects of this pernicious habit and have been adopting measures to discourage its use as an intoxicant. To meet this object the cultivation of poppy heads was restricted after 1901 to the following places —

Name of place	District	Object for which cultivation was allowed
1 Thaneswar tahsil	Karnal	For poppy heads
2 Pehowa circle	Do	Do
3 Kulu subdivision	Kangra	For opium extraction
4 Hoshiarpur tahsil	Hoshiarpur	For poppy heads
5 Lahore tahsil		Do
6 Chunian tahsil	Lahore	Do
7 Kasur tahsil		Do
8 Amritsar district	Amritsar	Do
9 Shahpur district	Shahpur	Do
10 Jampur tahsil	Dera Ghazi Khan	Do
11 Rajanpur tahsil	Do	Do
12 Kotkhai tahsil	Simla	For opium extraction

The measures adopted for restricting production of the poppy by the Government succeeded in eliminating the habit to a considerable extent so much so that 8 years after i.e. 1909 the habit almost died out from all other parts of the province except Jullundur, Hoshiarpur and Ludhiana districts. In 1909, therefore, the growth of poppy for poppy heads was further restricted to Jullundur district and Hoshiarpur tahsil only because of the high incidence of consumption which still existed in these districts. The area under poppy cultivation in these two districts is, however, very small. In Hoshiarpur district in 1926-27 there were only 304 acres under poppy cultivation and in Jullundur district only 512 acres. The total yield of poppy capsules from both these sources amounted to 224,760 seers or about 149,520 lbs. Only a small portion of this produce is used for medicinal purposes, practically the whole of it being consumed for euphoric purposes. In Simla and Kangra districts and in the Hill States although poppy is largely grown the use of the capsules as an intoxicant is practically non-existent. Excise records show that in Simla district the consumption of poppy heads during 1927-28 was only about 14 seers and that was chiefly for medicinal purposes. Even in the Hill States where the cultivation of poppy and extraction of opium from it cannot be so strictly controlled, no cases of this addiction were met with. In the two districts above named the use of poppy heads for euphoric purposes has lingered while it has almost entirely disappeared from other parts of India. Even in the adjacent districts, i.e., Ludhiana, Gurdaspur and Amritsar, addiction to this drug is comparatively very small.

As regards the control exercised by the Excise authorities on its production and consumption, it should be made quite clear here that nobody is allowed to cultivate *post* unless he has obtained a licence from the Deputy Commissioner of the district. The licence is entered on the *Patwar*'s (Revenue Official) register who inspects the field very frequently during the season. During the harvest season the produce is collected and weighed in his presence and entered in his register. Out of this produce the cultivator cannot sell a single poppy head himself. He is only allowed to sell the produce to the wholesale vendor who is a licensee of the Government. He keeps a stock and supplies the retail vendors according to their needs. No individual is allowed to possess more than two seers (4 pounds) of poppy heads and the licensed vendor is forbidden to sell more than this quantity. The export and transport of the poppy heads is not permitted without a pass issued by the local authorities. The wholesale and retail dealers must obtain a licence if they wish to transport poppy heads from one part of these districts to another.

It will thus be seen that the Punjab Government is exercising a strict control over the production, sale, possession and consumption of the poppy heads. Their price is being gradually increased by raising the acreage rate levied by the Government. In 1915 it was Rs. 4 per acre which was increased to Rs. 8 per acre in 1926 and further increase is being contemplated.

*Present extent of poppy addiction*—Our inquiries show that the only parts of India, where the poppy heads are used for euphoric purposes and where we found

persons habitually addicted to them at the present time, are certain districts in the Punjab and parts of Rajputana States, especially Jaipur. In other parts of India one does meet cases here and there but the addiction seems to have disappeared. During the course of our investigation of the opium habit in the central districts of the Punjab, we came across such a large number of these addicts that we thought it worth while to study it in detail.

We have tried to gauge the extent of this addiction in the Punjab generally and in the central districts particularly. A reference to Table I will show that consumption of poppy heads did not show any great fluctuations between 1901 and 1922, but in 1923 the consumption was about double of what it was in the previous year and in 1924 it increased still further. In 1925 and 1926 there was a slight decrease from the figures of 1924, but these still show an increase of about 70 per cent over those for 1922. We know that poppy heads are not commonly used for any purpose other than for addiction and the consumption for the medicinal purposes is negligible. It is not clear why the consumption of poppy heads showed such a marked increase during these particular years. Is it possible that the greater demand for this commodity was due to the great rise in the price of opium which has been gradually increasing? This is supported by the fact that when poppy crop is not good and the prices run high, the addiction to poppy heads shows a fall and consumption of opium slightly rises in these areas.

The habit is somewhat more prevalent among the urban areas than among the rural population. The city of Jullundur and the adjoining villages show the greatest incidence. One shop in the city alone showed a sale of 29,616 *seers* (59,232 lbs) in the year. There are quite large rural areas in these districts which are quite free from addiction. An addict seems to act like an infective agent and spreads the habit to others who come in contact with him. In the villages we were able to trace the spread of the habit to association with and on the advice of other addicts who described the wonderful effects of the drug in all sorts of conditions.

As regards the number of addicts it is very difficult to form an exact idea, as there is no system of registration of addicts. It is, however, possible to form an approximate idea of their number from the total amount of poppy heads consumed in the province, and the average dose which we have been able to work out from the series of 530 cases which we have studied. This is about 1.5 *chhataks* (about 3 ounces) per head per day. In the year 1925-26, 204,752 *seers* of *post* were consumed in the Punjab. Assuming that the whole of the amount was used for euphoric purposes, the number of addicts could not be more than about 6,500 in the whole of the province. According to the same calculation Jullundur has not more than 4,000 addicts, Hoshiarpur not more than 2,000, Ludhiana not more than 300 and Gurdaspur not more than 200. The actual number is probably somewhat higher in these areas as a certain amount of poppy heads are very likely kept by the agriculturists for their own consumption and are not returned in the Excise Statistics. Table II gives the figures of the consumption of poppy heads in different districts of

the Punjab It will be observed that with the exception of Hoshiarpur, Jullundur, Ludhiana and Gunderpur the amount of poppy heads consumed are negligible

TABLE I

*Poppy cultivation for capsules exclusively Area in acres and the quantity of poppy seeds sold by the cultivators to licensed vendors*

Year	Area in acres that came to maturity	CONSUMPTION OF 'POST' IN THE PUNJAB	
		In seers	In lbs
1901	874-2-32	150,139	300,278
1902	612-1-1	151,959	303,918
1903	533-0-26	141,134	288,268
1904	813-3-3	130,604	261,208
1905	528-2-29	131,115	262,290
1906	848-0-1	143,121	286,242
1907	563-2-13	109,856	219,712
1908	626-2-27	109,281	218,562
1909	398-1-26	108,212	216,261
1910	367-1-39	105,670	211,340
1911	518-6-0	124,606	249,212
1912	244-4-35	126,340	252,680
1913	448-4-18	124,860	249,720
1914	481-6-1	135,150	270,300
1915	158-4-26	84,116	168,232
1916	503-1-18		
1917	395-4-20	142,991	283,982
1918	244-1-27		
1919	356-5-11		
1920	469-3-15	114,948	229,896
1921	499-4-21	132,036	264,072
1922	328-3-4	120,687	241,374
1923	972-4-5	239,707	479,414
1924	703-1-14	273,782	547,564
1925	695-2-17	204,752	409,504

## TABLE

## Consumption of 'Post'

Name of the

H  
P

1925-26

121

20

55,434

131,604

11,012

1,452

590

1,438

2,717

301

515

167

70

58

117

70

75

34

79

389

56

47

8

108,232

84,116

114,948

204,752

= 2 lbs approximately

Since there is a large Sikh population but the addiction is not confined to this class only, it is also prevalent among the other communities residing there. It is usually the lower grades of society, however, who use it.

## PREPARATION OF THE BEVERAGE AND MODE OF ITS CONSUMPTION

The addicts are very fond of the company of other addicts and waiters who generally seek solitude in order to indulge in their craving. Drinkers seek every opportunity to take their beverage in company. For this purpose they assemble at specified hours at specified places in the morning, afternoon and evening for taking the beverage. As a rule are unfrequented spots such as a clump of trees, a well, etc.

abandoned place of worship or something of that nature. There they can unmolested and unknown to their relatives who might stand in the way of taking the drug. As the place gets known to addicts it attracts more and more. Such places are usually called *Dara* but in Jullundur, where the addicts are generally low class Mohammedans, these places are also called *takia* or *akikhana*. There are a number of them in Jullundur city and in many of the villages in that district, which have been kept going for years. Generally there is a man who either lives in those drinking places or at least spends most of his time there to look after the establishment. This person is called *saki* and as a rule he is an indigent person or a beggar who has been an addict most part of his life. He may be a Hindu or a Mohammedan according to his clientele, when the latter, he is a *sain* and is generally addressed as *ustadji* or a master. A *saki* or *sain* is generally an old emaciated pale-looking and anæmic individual. He sits on a dirty piece of matting with a number of earthen bowls in front of him, with quantities of soaked poppy heads in them. The capsules he buys at about 3 pice per *chhatack* (2 ounces) and sells them at 8 pice a *chhatack* after preparing the potion from them, thus making a little profit. He may supply ready made *post* to the addicts on payment or he may be asked merely to supply an earthenware bowl and water and a coarse piece of cloth for straining the drink to those who bring their own capsules. Poor people as a rule buy their dose every time they drink it, but those who can afford, buy the maximum quantity allowed under Excise regulations and use the required quantity out of it every day. The dosage is calculated by the number of capsules and is usually 5 to 6 capsules at a time. The addicts put the crushed capsules in a bowl half full of water (6 to 8 ounces), sit down and watch them till they become quite soft. The capsules are porous and water quickly penetrates and softens them. As a rule, 20 minutes to half an hour suffice for this purpose. The softened pieces are then broken down into a fine pulp by rubbing them with fingers and squeezing out the juice. This process appears to be very much enjoyed by the addicts and some of them spend one or two hours at it. It is generally accompanied by long whiffs of tobacco-smoke through a *hubble-bubble* which is passed round, among a number of them all engaged in the same occupation. In summer it suffices to soak the crushed capsules for not more than an hour, but in winter they are not infrequently soaked overnight. Sometimes the capsules are put in whole without crushing and are thoroughly macerated with hands after they become soft. Whatever method is adopted the alkaloids present in the poppy heads gradually pass into solution in water and the process of rubbing with hands and fingers completes the extraction. When this process is completed the mass is strained through a coarse cloth till all the juice is squeezed out. The residue is again rubbed with a little more water and again strained. This process is repeated 2 or 3 times till the addict is convinced that there is no more of the active principles left in the residue. The beverage thus prepared is known as *post* and is drunk immediately after preparation. It is a pale yellowish looking fluid having a bitter taste and a peculiar faint aromatic odour. Most of the addicts take it in

the morning and in the afternoon but some take it 3 or 4 times a day or even oftener. The solid residue left in the cloth is called *pichhar* which is generally thrown away but is sometimes re-extracted by the poorer addicts who cannot afford to buy poppy heads. Small amounts of the intoxicating alkaloids are undoubtedly left behind even after a thorough rubbing. The drink usually takes 20 to 30 minutes to produce its effect and is frequently followed by a little tea with sugar or powdered ginger or cardamoms. It is believed that these accessories, especially the sugar, enhance the effects produced by the drug. The addicts generally remain in the *takia* for a long time, after they have taken their potion and enjoy each other's company. They sit down in groups, some of them enjoy a smoke, others amuse themselves by playing cards or watching cock fighting or quail fighting.

During the process of separation of the poppy seeds from the capsules a lot of broken bits of the capsules are separated which are known by the name of *phal*. This is sold at a cheaper rate than the whole capsules and is rich in alkaloidal contents. Poor people who cannot afford to buy capsules, buy it and prepare their beverage from it in the same way as *post*.

Besides the process of extracting the active principle by macerating the capsules in water which has been above described, the addicts sometimes boil the capsules in water and drink the infusion. Of late years capsules have been boiled with tea and the resulting beverage is taken with sugar and milk in the usual manner. In these districts we found tea infused with poppy capsules was being sold to the addicts probably without the knowledge of the Excise authorities.

Green, ripe capsules are sometimes used, they are fried in butter or *ghee* (clarified butter) and are eaten by the addicts. This preparation is known by the name of *bhujji* and is believed to have similar effects as *post*. Sometimes the juice of green poppy heads is extracted and a sweet called *halua* is made from it. This preparation is very soothing and is said not to have any constipating effects.

#### CHEMISTRY OF POPPY CAPSULES

Before describing the symptoms and effects produced by poppy heads it will not be out of place here to say a few words about the chemical composition of the capsule from which opium has not been extracted. A perusal of the literature shows that no detailed analysis of these has been done, since the alkaloids obtained are so small as to be of no commercial value. Lyon of Bombay (1879) analysed poppy capsules from Malwa. He obtained from them 0.099 per cent of the alkaloids soluble in ether, consisting apparently of narcotine, 0.023 per cent of impure alkaloids soluble in benzol, and 0.033 per cent of impure alkaloids soluble in chloroform. No morphine could be detected by the ordinary reagents. The Department of Chemistry at the School analysed the capsules at the request

of the senior author and we are very grateful to Mr N Ghosh for the following results —

One hundred and thirty-seven grammes of the powdered capsules yield 15.18 grammes of the *alcoholic extract*. This contained 1.05 grammes of the total alkaloids of which only 0.015 per cent (or 0.0015 grams) was morphine. About 50 to 60 capsules weighed 137 grammes and contained a total alkaloidal content of 1.05 grammes. The average dose is 5 to 10 capsules which will mean about 1 to 2 grams of the alkaloids in each dose or 3 to 5 grams a day. This quantity would be quite sufficient to produce the effects for which the poppy heads are taken.

A larger quantity of capsules sufficient to obtain 30 grammes of the alkaloids were extracted. The quantities of the chief alkaloids which were detected is given in the following Table —

TABLE III

Name of alkaloid	From 3 grammes of total alkaloids	Percentage
Narcotine and papaverine	0.92	30
Codeine	0.78	26
Morphine	0.14	4.5
Other alkaloids and impurities	1.16	39.5

It will be seen from these results that the poppy capsules contain a very small quantity of morphine and large quantities of narcotine, papaverine, codeine and other alkaloids which have a decidedly weaker depressant action on the central nervous system.

As regards the keeping properties of the poppy capsules the general idea prevalent is that they deteriorate with age. The addict always prefers the fresh poppy heads as they produce better effects and for medicinal purposes also old stocks which have been lying for a year or two are not recommended. We are carrying out a series of observations to determine the variations in the alkaloidal contents of the capsules produced by age and these will be published in due course.

#### AN ANALYTICAL STUDY OF 530 CASES OF ADDICTION TO POPPY CAPSULES STUDIED

We have failed to find any reference to this addiction in the medical literature although it would appear to have been fairly widespread in India not very long



TABLE IV

Analysis of 530 'post' addicts according to doses

Doses	A					B							C							D						
	CAUSATION					DURATION							AGE TO START							PRESENT AGE						
	Association	Disease	Hard work	Pleasure	Substitute for alcohol	1 year	2 years	3 years	4 years	5-10 years	11-20 years	Over 20 years	Under 20 years	21-30 years	31-40 years	41-50 years	51-60 years	61-80 years	Over 80 years	Under 20 years	21-30 years	31-40 years	41-50 years	51-60 years	61-80 years	Over 80 years
Up to 1 ch	71	130	13	31	30	6	14	8	19	96	77	55	64	88	75	75	5	8		5	40	79	65	43	43	
Up to 2 ch	52	56	7	26	28		3	3	6	50	55	52	59	72	31	3	1	1		1	35	51	39	21	21	2
Up to 3 ch	16	13	1	7	2	1	1			18	12	14	20	16	2	1				11	14	5	6	3		
3 ch and above	16	15	3	12	2		2	2	1	11	15	19	21	17	5	4	1			1	5	12	8	15	6	
Total	155	214	24	76	62	7	18	13	29	165	159	140	163	193	113	45	7	9		7	91	156	117	85	73	2
Percentage	29.2	40.3	4.5	14.1	11.7	1.3	3.4	2.4	5.4	31.1	29.8	26.4	30.7	36.4	21.3	8.5	1.3	1.6		1.3	17.1	29.4	22.0	16.1	13.7	0.18

TABLE IV—*concl'd*Analysis of 530 'post' addicts according to doses—*concl'd*

Doses	L			F																C								
	EFFECT AS JUDGED BY ADDICT			Occupation																SEX								
	Beneficial	Harmful	Total	Weaver	Leather worker	Household	Merasi rubber	Agricultural	Shopkeeper	Barber	Palinist	Tin solderer	Sadhu	Tailor	Artisan	Service	Labourer	Tonga driver	Chaukidar	Chick worker	Habam	No work	Milkman	Butcher	Prostitute	Male	Female	Total
Up to 1 oh	102	113	215	2	17	10	14	73	32	5	2	6	53	10	24	14	10	5	4	4	2	13				208	7	215
Up to 2 oh	38	71	109	6	11	3	8	56	19	5	1	1	14	1	12	1	5	3	5	2	1	1		1	1	166	3	169
Up to 3 oh	17	22	39	2	3		2	13	2	1			5		3	2	2	1				1		1		38	1	39
3 oh, and above	16	31	47		1	3	3	16	4	2			8	2	1	3	1					1			2	43	4	47
TOTAL	203	237	530	10	32	16	27	154	57	13	3	7	50	10	40	23	27	9	9	6	3	16		5	2	515	15	530
	553	147	100	18	60	30	51	287	107	24	6	13	94	30	73	43	50	17	17	12	00	30		00	04	971	28	100

ago It is probable that it was considered to be practically the same as opium addiction and no one thought of studying it separately We realized in the early part of our investigation that the two addictions differed in many respects A perusal of the section on the chemistry of the poppy capsules will show that their composition differs from that of opium The alkaloids belonging to the morphine group and morphine itself occur in much smaller quantities than those of narcotine group The effects on the individual, therefore, would differ

(1) *Type of addicts*—The addicts generally belong to the lower grades of society, the majority of them having very meagre means Those belonging to better classes are few in number and are generally those individuals who have tried all narcotic drugs such as hemp drugs, opium, alcohol, cocaine, etc An inquiry made in these two districts showed that in Jullundur district, where the Mohammedan population is large, most of the addicts are Muslims belonging to Rangpur and Rajput classes whose main occupation is agriculture In Hoshiarpur district the Sikh agriculturists predominate In both these districts a large number of addicts belong to the menial classes such as sweeper, water-carrier hackney carriage drivers, etc No particular cause can be assigned as to why they become addicted to poppy heads except that they are obtainable here and that they are somewhat cheaper than opium The average daily dose of this drug works out at about 1 anna while that of opium about 2 annas a day

(2) *Dosage*—The average dose of poppy heads estimated from our series of 530 cases worked out to be  $1\frac{1}{2}$  *chhatacks* (or 3 ounces) per day This may be taken in at once, but generally is divided into 2 or 3 portions and taken morning and evening or in the afternoon also About 8 to 10 average sized capsules weigh about 1 *chhatack* A capsule when lanced gives about half a gram of opium on an average but we have already pointed out that when the capsules, from which opium has not been extracted, are ripened and dried they neither yield the same quantity nor the same quality of the alkaloids An average dose of poppy heads, however, contains sufficient alkaloids to produce the effect which the drug produces We came across some addicts who took as much as 8 to 10 *chhatacks* a day but this was very rare As a rule the dose was kept below 5 *chhatacks* (10 ounces), by far the large majority taking between 1 to 2 *chhatacks*, equivalent to 3 to 6 grains of the alkaloids

### (3) *Causation of addiction*—

(a) *Disease*—A perusal of Table IV, A, will show that in our series as many as 40·3 per cent took to this drug for some disease or ailment which it was supposed to cure It is interesting to note that out of 214 cases who took the drug for some malady the majority, i.e., 47·2 per cent kept to very small doses of under 1 *chhatack* (2 ounces), 39·5 per cent took between 1 to 2 *chhatacks* and the remaining took larger doses Table V gives a resumé of the different diseases and ailments for which the drug was taken

TABLE V

*Statement showing various diseases for which 170 addicts started the 'post-addiction habit*

Diseases	½-1 ch	1-2 ch	3 ch	4 ch and above	TOTAL
Diseases of eyes	40	10	5	2	57
Cough and coryza	14	11		1	26
Hæmoptysis	4	2	3	1	10
Asthma	11	2		1	14
Bowel diseases	7	2	2	1	12
Piles	1	4		1	6
Backache	3	10		5	18
Pain in chest	1			1	2
Sciatica	2	1		1	4
Rheumatic pains	1	1			2
Caries bones		1	1		2
Sinus jaw		1			1
Injuries	3	1			4
General tonic	2	1			3
Enlarged spleen			1		1
Palpitation of heart		1			1
Urticaria			1		1
Insanity		1			1
Gonorrhœa	1		1		2
Syphilis	1				1
Spermatorrhœa			1		1
Dysuria				1	1
TOTAL	91	40	15	15	170

It will be observed that eye diseases come first on the list, then come diseases of the respiratory system such as asthma, hæmoptysis, cold, cough, etc., next follow aches and pains and then the bowel diseases. It is evident that poppy heads have never been taken for any serious disease.

(b) *Association* —The next important cause of addiction appears to be association. We have already mentioned that *post* addicts are very fond of company. They always like to have friends or associates around them and hence they collect in *takras* for taking the drug. They advise *post* as a panacea for all the ailments and troubles. Most of the people get the habit by advice and even entreaties of the addicts. We made a special study of 420 cases with a view to see if the addiction could be hereditary and came to the conclusion that heredity did not play any part. We were, however, struck by the fact that 32.2 per cent of the addicts gave a history of addiction in some member or other of the family. This is evident from Table VI. In 17.0 per cent of these one of the parents was an addict. Out of the whole series 155 or 29.2 per cent contracted the habit by association. It will be observed that in this group there is a tendency to larger doses.

TABLE VI

*Effects of heredity*

Doses	Father	Uncle	Mother	Grand father	More than one member	None	TOTAL
Up to 1 ch	30	5	4	9	16	144	208
Up to 2 ch	22	1	2	7	15	92	139
Up to 3 ch	4		1		3	25	33
3 ch and above	9	1		2	4	24	40
TOTAL	65	7	7	18	38	285	420
Percentage	15.4	1.6	1.6	4.2	9.0	67.8	100

(c) *Euphoria* —The next in order come the pleasure seekers who form 14.1 per cent of this series. Among this group are included those individuals who take it entirely for its soothing and euphoric effects. They are generally young people who have ample means and have little to do, they are fond of company, are ease-loving and spend most of their time in enjoyment and recreations peculiar to their class, e.g., cock-fighting, quail-fighting, etc. They rapidly increase the dose and take large doses of the drug.

(d) *As a substitute for alcohol or for hard work* —There are only 11.7 per cent who take this drug as a substitute for alcohol and they are as a rule satisfied with small doses, sufficient to minimize the craving they have for liquor. The majority of this group were Sikh agriculturists who, having taken to drinking large quantities of alcohol, find it too expensive and take to this drug as a substitute. Only a small number 4.5 per cent take it to enable them to bear the strain of hard work.

(4) *Duration of addiction* —It will be seen from Table IV, B, that only in 12.5 per cent of the cases the duration of habit was under 4 years, in 31.1 per cent for 11 to 20 years and 26.4 per cent over 20 years. It follows, therefore, that the habit once started lasts for considerable periods and is very difficult to break.

(5) *Age when started* —A perusal of Table IV, C, will show that in the majority of cases the habit was started in early adult life. In 30.7 per cent it was started below the age of 20, in 36.1 per cent between the ages of 21 and 30, in 21.3 per cent between 31 and 40 years, a very small number, i.e., 11.5 per cent of the total developed the habit after 40 years of age. Of the small percentage who started the habit after 40 years of age the majority found it necessary to take it for amelioration of some ailment or disease. Pleasure, hard work and substitute for alcohol find no place among the addicts who take it after the age of 40.

(6) *The present age of addicts* —Regarding the present age of addicts a perusal of Table IV, D, will show that only 1.3 per cent of our series were below the age of 20, 66.5 per cent being between the ages of 21 and 50. An analysis of our cases shows that the younger men become addicted to the habit chiefly for its euphoric effects and as a substitute for alcohol and lastly to enable them to bear the strain of hard physical work.

(7) *Addict's idea of its effects* —As regards the effects produced upon an addict it will be seen from Table IV, E, that 55.3 per cent of the addicts thought it had beneficial effects on them, while 44.7 per cent considered it was harmful. As a rule the addicts taking small doses claim that it does them good, generally by keeping in check such minor ailments as cough, cold, aches and pains, watering of the conjunctiva and other worrying symptoms, from which they suffer. In this series we found that with small doses such as under a *chhatuck* (2 ounces) a day there are no apparent ill-effects either physical or mental. With bigger and more frequent doses it is decidedly harmful.

(8) *Occupation of addicts* —A perusal of Table IV, F, will show that the habit is universal among the menial classes. The agriculturists take it to enable them to do hard work. Those having sedentary occupations and have to sit for long hours, e.g., shopkeepers, weavers, tailors, etc., form quite a large group, whilst 16 per cent constitute the no-work class who are either vagabonds, beggars, etc., or who have to wander about to get a precarious livelihood. There were five prostitutes in our series who said that it is useful for leucorrhoeal discharges and professed that it keeps their genitals dry and enhances the sexual act.

(9) *Sex* —A perusal of Table IV, G, shows that 97.1 per cent of the addicts studied were men and only 2.8 were females. This corresponds more or less to what we found in opium addiction and the same reasons hold good here.

#### EFFECTS PRODUCED BY HABITUAL USE OF POPPY HEADS OR 'POST'

We were unable to find in the medical literature any account of the symptoms and effects produced by the use of poppy heads on those who habitually indulge

in them The following description has been compiled from a careful analysis of our series of 530 addicts we have studied

The symptoms and effects produced by opium and poppy heads differ more in detail rather than in their general aspect These naturally differ with the dose taken, duration of the habit and individual idiosyncrasy On the whole it may be said that the effects of the capsules are milder but not so lasting as opium, as the former drug has to be taken more frequently A perusal of Table III shows that poppy heads contain morphine and codeine and narcotine and papaverine in about equal proportions but the amount of morphine itself is very small as compared with that in opium Although most of the opium alkaloids depress the psychical areas the action of morphine in this respect is much more powerful and the same is true of its analgesic effects In fact papaverine, narcotine and also codeine to some extent, act more as excitants than depressants and this stimulant action especially on the psychical areas appears to be a prominent feature of *post*

Within a few minutes after taking his potion the addict begins to show signs of ease, comfort and a general feeling of well-being There must be some psychical element in this as the alkaloids would probably take 10 to 20 minutes at least to be absorbed from the gastro-intestinal tract into the circulation in sufficient quantities to produce their effects There is undoubtedly a marvellous change in the addict soon after the potion is drunk From a condition of lethargy, fretfulness moroseness and peevishness, he passes into a state of gaiety and talkativeness He looks happy becomes very communicative and companionable This state of affairs lasts for  $1\frac{1}{2}$  to 2 hours and gradually the agreeable feeling of elation passes into a state of depression, the individual becoming drowsy and may fall off to sleep The stage of depression is not nearly so marked as in the case of opium The effects completely pass off in 5 to 8 hours The excitement stage, we have already said is more pronounced than in case of opium The effect appears much more quickly, probably due to the fact that the alkaloids are taken in form of solution and are absorbed more rapidly When the habit has established itself for a long time the addict generally looks dull and sleepy, becomes slow of comprehension and inattentive His gait becomes heavy, his movements slow, he is careless in dress and dirty in his habits His speech is slow and hesitating, in monosyllables jerky and his voice is husky as if he is talking in sleep The only time when he brightens up and looks his normal self is when he has taken his potion, and for 2 or 3 hours afterwards Even then his method of talking gives him away He speaks as if in a dream, he pays little attention to what is said to him but goes on muttering to himself The special senses are not directly affected but appear to be disturbed through impaired attention or heightened reflex irritability

Even small doses, e.g., 5 or 6 capsules a day appear to produce a marked physical deterioration when continued for prolonged periods and the addict becomes mentally degenerated and lazy Probably the chronic constipation which is present in the majority of these individuals and the consequent intestinal toxæmia

have a great deal to do with it. We found that even those addicts who took small doses could carry out their ordinary vocations only with difficulty.

The addicts say that poppy heads do not upset their digestion, in fact they claim that it sharpens their appetite and they can eat more and digest better. They claim that their eyes feel dry, the sight is improved and cough and expectoration are decreased. It is said to dry all the excessive secretions. Some claim that it gives them relief from asthma. A drink of *post* in the evening after a hard day of toil refreshes them and gives them ease of mind and languor of the body.

Those addicted to this drug are generally believed to suffer from sluggishness of the bowels and chronic constipation. It is well known that morphine decreases general sensitiveness and, therefore, responsiveness to the defaecation act. The response to distensive stimulus of peristalsis is decreased and there is quietening of peristaltic movements. Opium, therefore, gives rise to chronic constipation. The action of morphine esters, e.g., codeine is less marked in this respect and that of narcotine and papaverine is even weaker. These latter alkaloids, however, have a direct, depressant action on the smooth muscle of the intestine and therefore tend to diminish peristalsis. The act of defaecation thus becomes difficult, so much so that the addicts sit for hours together and forget that they are in the act of defaecation. Constipation does not appear to be so common in these addicts as in opium addicts. Out of 221 addicts in our series, in which we specially studied the effect of the drug on the bowels, only 32 complained that they suffered from it badly. The drug undoubtedly has a well marked diuretic effect and those addicted to it micturate very copiously and frequently.

The addicts are very forgetful not only of themselves but of their own surroundings. They appear to lose all idea of correlation of time and space, they forget their environments and do not know what they are engaged in doing. They may sit in one place for hours together doing nothing without feeling it. They may walk a few yards and think that they have travelled for miles or they may have walked for miles and think that they have walked only a few paces. They may go on doing hard work for hours without feeling it or they may sit idle for long periods. They lose the idea of correlation of touch perception and localization and many stories are told about it\*. The addict to *post* becomes mechanical or automatic in his actions and appears to have no control over his will power. Once he starts doing a thing he will go on till he is reminded that it was time he stopped.

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\* Some *postis* were sitting near each other when one of them had the desire to scratch his leg. He began to scratch his neighbour's leg thinking that that was his own. Another story related is that a number of addicts were sleeping near each other, as is their custom. One suddenly woke up and saw his own fixed knee up before him. He thought it was the head of a thief and he struck a hard blow at it and shouted 'I have hit the thief's bald head but he has struck a blow at my knee when running away'.



## PHYSICAL, MENTAL AND MORAL EFFECTS

That addiction to poppy heads produces considerable physical, mental and moral degeneration there is little doubt. Our impression from the study of our cases is that as compared with opium these effects are much more pronounced with much smaller quantities of the alkaloids which are consumed in the poppy heads. The features we have described above are so typical of the addict that he can be easily recognized. While an opium addict taking small doses may not be easily differentiated from a normal individual, a person taking even small quantities of *post* at once gives himself away. So much so that in the Punjab a lazy, slovenly, dull and unintelligent-looking person is often called a *postri* (or one who indulges in poppy capsules).

The addicts who have taken the drug for some time are, as a rule, spare and emaciated individuals with stunted growth and sub-normal weight. They have a sallow, muddy appearance, sunken eyes and cheeks and are very anæmic. Their eyes look dull and sleepy, they have heavy palpebræ and dry conjunctivæ. Advanced cases who have taken large doses look cachectic, have a dirty tongue, foul breath and give the impression of suffering from chronic intestinal toxæmia. The subcutaneous fat is absent and the muscle tissue is wasted so much that the dry skin becomes quite loose over it. The throat is dry, respirations are slow and shallow and the expansion of the chest is impaired. The pulse at the wrist is weak, slow and compressible, soft hæmic murmurs are not unfrequently audible.

As regards the mental effects they differ somewhat from those of opium. The excitement stage is more prolonged on account of the smaller amount of morphine and larger quantities of the alkaloids of the iso-quinoline group, i.e., narcotine and papaverine. There is a feeling of elation, exuberance and well-being, which manifests itself in speech and gestures. In this stage the addicts become very communicative and reveal all their secret thoughts. There is loss of responsible control over mental processes, but the control of movements is not impaired as is the case with alcohol. The net result of the action on the psychic areas is unrestrained imagination which may take different directions in different individuals. In some it will produce excitement, in others drowsiness and sleep. The irritation of the nerve cells produces hallucinations which, though present in this addiction, are not so prominent a feature as in case of cocaine. The effect on the addict who has taken the drug for long periods resembles a chronic poisoning of the nervous system especially of the higher psychical areas, which alters the mental activity from a state of high irritation to a complete breakdown even to paralysis. The individual becomes dull, lazy and careless, he has a vacant look, and his eyelids droop, the temper is often irritable and he gets excited quickly, his will power is weakened and he loses his determination of mind and character. The addicts are generally feeble-minded individuals and are untruthful, selfish and self-centred.

As regards the connection between insanity and addiction to this drug no distinct relationship could be traced. Four out of the 530 cases studied were

insane and in two of these at least the drug had been taken for prolonged periods and in large doses

*Abstinence symptoms* —These closely resemble those of opium. The effect of one dose of *post* lasts for 4 to 8 hours and it is generally so arranged that by that time the next dose is taken. Usually about half an hour before the due time the addict begins to yawn, feels dull, heavy, depressed and very tired. His eyes begin to run there is sneezing, salivation and frequent spitting. Some have flatulence, eructations, and even pain in the abdomen. If a dose is not then taken, the fatigue increases, he gets headache, vague pains all over the body which may localize themselves in some of the joints and may become excruciating. If still the dose is withheld the restlessness increases, the addict becomes very irritable, peevish and restless, he becomes morose and despondent of his life, he has a sinking sensation and there may be a feeling of impending death. In some individuals the respiratory symptoms are more marked, the addict suffering from dyspnoea, and paroxysms of severe cough. In others the gastro-intestinal symptoms are prominent, there being frequency of stools, diarrhoea and colicky pain. In yet another group the nervous symptoms are the chief manifestations, the addict complaining of dimness of vision, formication, tingling sensation over the body and insomnia. As a rule the addicts are so careful of their next dose that they do not give an opportunity for these symptoms to arise by making sure of it when it is due.

We have been told by addicts over and over again of the disastrous results which will accrue, if by any chance it is contemplated to stop the cultivation of the poppy in these districts at once and their supply of poppy heads is cut off. A study of the addicts has impressed us with the fact that the habit is deeply ingrained in them and any sudden withdrawal of the drug will give rise to acute distress among them. Registration of the addicts and such measures as have been introduced in some of the districts of Assam, together with a gradual, progressive reduction in the amount of poppy heads produced till their complete extinction, would probably be the best solution of the problem.

#### DIFFERENCES BETWEEN THE EFFECTS PRODUCED BY 'POST' AND OPIUM

We have already said that the symptoms and effects produced by *post* and opium bear resemblance but the excitement stage is considerably longer in case of *post*, in fact some of the effects resemble on a mild scale those produced by cocaine. Here we will confine ourselves to the addicts' own idea of the difference between the two drugs.

The common belief among the addicts is that *post* does less harm than opium. The reason given is that a person addicted to poppy heads requires a comparatively much larger dose of opium to produce the same effect. Opium is said to have a more constipating effect, it upsets digestion and produces loss of appetite. They also say that *post* is a cooling and refreshing drink, and quenches thirst, while

opium is heating For this reason many addicts take to poppy heads in the summer and opium in the winter They say that *post* is less intoxicating and its effects are milder but more prolonged They claim that it does not form a habit so readily as opium This, however, is not borne out by our observations Opium produces a peculiar muddy, sallow, coloration of the skin while *post* does not do this It is believed that when opium is being taken it is necessary to take rich food such as sugar, milk, ghee, etc., but poppy heads do not require these accessories They are, therefore, preferred by the poorer classes Another reason given for preference to poppy capsules is that opium is often adulterated but the capsules are always free from this defect Besides this the capsules are said to have diuretic properties while opium is not considered to be good for the kidneys It is said not to have the same aphrodisiac action as opium

#### SUMMARY AND CONCLUSIONS

(1) Poppy capsules were used for medicinal purposes in the early classic Greek and Roman periods They have also been used for many centuries in the Chinese medicine in the treatment of bowel diseases and in the Hindu and Mohammedan medicine in India against diseases of the alimentary and respiratory tracts and painful inflammatory swellings of the body

(2) The use of poppy capsules for euphoric purposes dates from the very early times In India this was started soon after the introduction of poppy cultivation into the country References to the Moghul literature show that a beverage prepared from poppy heads was extensively used all over India in the 16th, 17th and 18th centuries and later

(3) Since the introduction of restrictions in the cultivation of poppy, the temptation has been removed from the peasants' door and the habit has practically disappeared from most parts of India

(4) The only part of the country where poppy heads are used, at the present time for purposes of addiction, are some of the districts of the Punjab, chiefly Jullundur and Hoshiarpur and some of the Rajputana States The total number of addicts in the former area is probably not more than 6,500

(5) The preparation of the beverage and the mode of indulgence is described.

(6) A chemical analysis of poppy capsules from which opium has not been extracted has been carried out The results show that, as compared with opium, they contain much smaller quantities of morphine and much larger quantities of codeine, narcotine and papaverine

(7) These latter alkaloids have a weaker effect on the psychical areas and a weaker analgesic effect, in fact as excitants The symptoms, therefore, differ somewhat from indulgence, and resemble in some respects those produced by much milder scale

(8) An analytical study of 530 cases of addiction to poppy heads or *post* has been carried out, and different factors in connection with its causation, dosage, symptoms and effects produced have been recorded

We are very grateful to the Governing Body of the Indian Research Fund Association who have been good enough to finance this research

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# ON TRAPPING ADULT MOSQUITOES

BY

C STRICKLAND, M A , M D ,

*Professor of Medical Entomology, School of Tropical Medicine, Calcutta,*

AND

K L CHOWDHURY, M B ,

*Malariaologist, the Endowment Fund, School of Tropical Medicine,  
Calcutta \**

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WHILE engaged in a malaria survey of the Duars tea-gardens in North Bengal (Strickland and Chowdhury, 1928) we used two types of traps for catching the

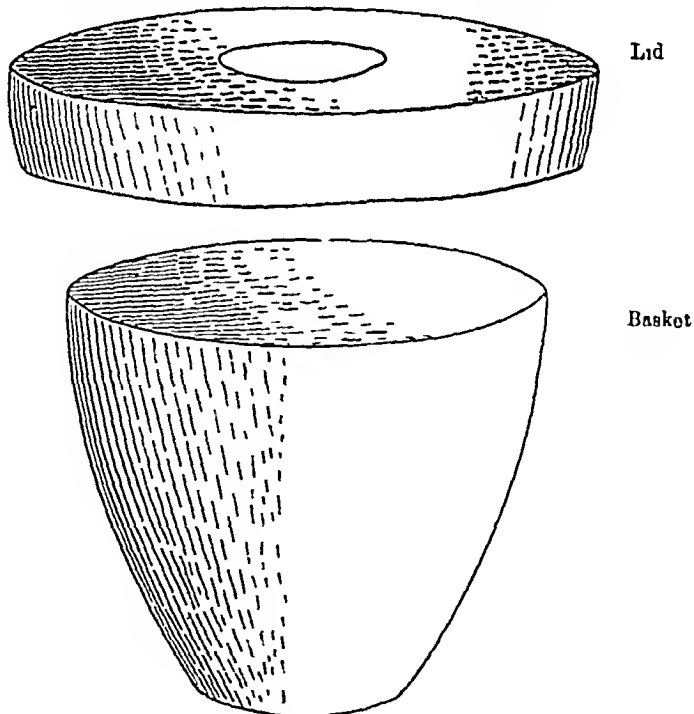


FIG 1

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\* We are much indebted to Mr McGregor for the care he gave to compiling the records used in this paper

adult mosquitoes The traps in question were described in the report of the survey (*op cit*) but may be shortly referred to here One was an ordinary tea-plucking basket 'leaped inside and out with cow-dung and covered with a lid (see Fig 1) with centre opening 6 inches in diameter—lid also leaped—hung up a little off the floor in a cowshed or other building, and a little grass or hay placed at the bottom of the trap' (Clemesha) the other an ordinary tea-box (Fig 2) about 18 inches each way,

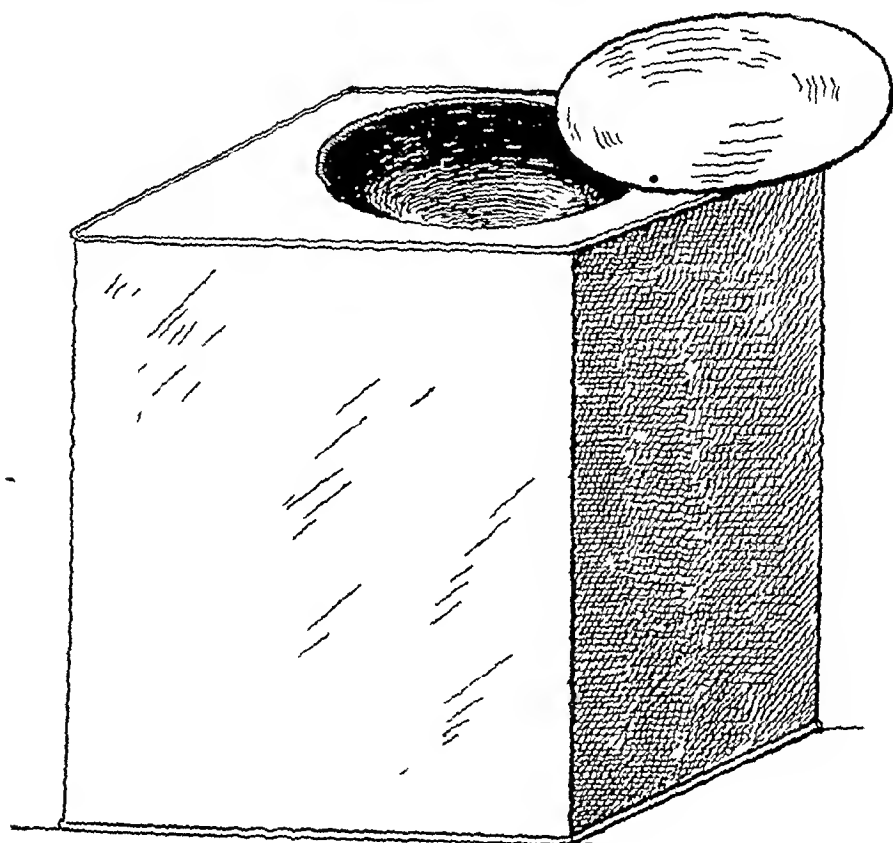


FIG 2.

and in one side of it a circular hole that could be closed by a swivelled door, the inside of the box being blackened with stencil ink

In that report it was stated, 'short of a test with the traps side by side conclusions as to their relative efficacy must be fallacious and will not be attempted' and that in a test it would be more important to know the better trap for catching the 'carrier-species' than all species

The following tables are the result of such a test with one trap of each type side by side which was made during a subsequent malaria survey in Upper Assam, the comparative number of mosquitoes caught in the two types of trap being shown in the tables

The survey extended over a period of two years, 1927 and 1928, but covering only the eight months of the hot weather and the rains each year. Only in 1927 was the culicine catch recorded

TABLE I

*All mosquitoes, all localities, 1927 survey*

Tea basket trap	Box trap
33,341	37,060

The box-trap was therefore slightly more efficient for the general catch

TABLE II-A

*Culicines, all localities, 1927 survey*

Tea basket	Box trap
30,878	34,000

The box-trap was the more efficient by about 10 per cent for catching culicines

TABLE II-B

*Anophelines, trapping in all localities, 1927 and 1928 surveys combined*

Basket	Box trap
8,157	9,207



The box was therefore the better trapper of anophelines by about 13 per cent

*Table II-B analysed*

The results of the analyses are tabulated below —

	1927	Per cent better	1928	Per cent better	1928 and 1929	Per cent better
Hot weather—						
Basket	490	10.0	52		542	.
Box	442	..	458	781	900	66
Rains—						
Basket . ..	1,973	.	5,642		7,615	
Box .	2,618	32.7	5,689	1	8,307	9
Hot weather and rains—						
Basket	2,463		5,694		<div style="border: 1px solid black; padding: 5px; display: inline-block;"> <p>Table II-B</p> <p>8,157    ..</p> <p>9,207    13</p> </div>	
Box .	3,060	24.2	6,147	8		

The more comprehensive of these analyses, viz, those showing the result (1) in the hot weather or the rains in the combined years, and (2) in 1927 and 1928 during wither season, unfortunately do not afford any verification of Table II-B, so that the finding given in this Table (II-B) cannot be definitely claimed to have been otherwise than possibly a chance one. If, however, this table shows correctly which is the better trap, then the annual and seasonal differences shown above in the subsidiary tables may have been due to some factors affecting the trapping and not necessarily to the errors of random sampling.

TABLE III

*Anopheline species all localities, 1927 and 1928 surveys combined*

Species	Baslet.		Box	
jamesi	7		7	
sinensis	496		578	
barburovris	2		17	
rossi ♀	3		18	
vagus ♀	4,071	9,070	4,746	11,027
rossi or vagus ♂	5,896		6,223	
culicifacies	39		169	
funestus	892	908	1,250	1,461
aconitus	16		19	
maculatus	107		64	
tesselatus	63		73	
jeyporiensis	0		2	
fuliginosus	362	476	517	6
philippinensis	114		154	
bochi	163		150	
gigas	0		1	

TABLE IV

*Sexual predilections of the anophelines for the traps**All anophelines, all localities, 2 years' survey**A Females*

Tea basket traps	Box	Box better by per cent
5,892	8,975	+52

*B Males*

2,265	2,260	±0
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The box type was more attractive to the females

The basket and the box were equally attractive to the males

These figures were not well supported by subsidiary analyses for the separate years or seasons and so they cannot be accepted as more than possible indications of the truth

## CONCLUSION

The so-called tea-box trap appears to be superior to the basket by about 12 per cent for catching both culicine mosquitoes and anophelines

Apparently the boxes were better than the baskets as far as nearly all the anopheline species including the well-known 'malaria-carriers' were concerned, *funestus* markedly favoured the tea-boxes

Females were caught very much more frequently than males by the boxes as compared with the baskets

In general all the above indications are in favour of the use of the box-traps, and not only so but these outweigh the baskets in all other advantages and it is therefore advised that they be generally used

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Thacker Spink, Calcutta

# THE DYSENTERIES OF MHOW, CENTRAL INDIA AND THE CENTRAL PROVINCES

BY

MAJOR C J H LITTLE, R A M C ,

AND

ASSISTANT SURGEON W BORNSHIN, I M D

[ Received for publication, September 12, 1929 ]

THIS report deals with an investigation, lasting from January 1926 to July 1928 into the causation of the dysenteries of Mhow, Central India, and of some neighbouring military stations in the Central Provinces. The work was preliminary to an attempt to produce a vaccine against bacillary dysentery which could be administered in some manner other than by subcutaneous inoculation, and was financed by the Indian Research Fund Association.

Cunningham, Acton and Knowles, and others, have shown that the idea at one time firmly held in India that almost all dysentery in this country is caused by *Entamoeba histolytica* is entirely incorrect. Manifold proved that, amongst the military and civil population of Poona, the dysenteries are almost all caused by *B. dysenteriae Flexner* and that a mild enteritis with dysenteric symptoms, known as 'Poonaitis,' is in fact bacillary dysentery.

Our own findings, so far as Mhow and neighbouring stations were concerned, confirm the above, for over ninety per cent of dysentery cases investigated were bacillary infections, by far the commonest organism present being *B. flexner*. The population from which the cases came was a military one, but, in order to ascertain if the same proportion held amongst civilians, we examined many stools from bazaar patients, and here again bacillary dysentery was far more commonly met with than amebic.

## COLLECTION OF MATERIAL

Most of the material examined came from the local garrison. The Central Provinces District Laboratory in Mhow suffers from being situated one mile from the British and over two miles from the Indian Military Hospitals. For some

months, all stools were collected from both hospitals in the bed pan and by motor ambulance, and the percentage of positive findings was over ninety. Then economy produced a sweeper on a bicycle, with material in test tubes, and the road to the laboratory is up-hill. Finally a sort of baker's hand-cart replaced the bicycle, with the advantage of being capable of carrying several bed pans, but with the drawback of being very slow indeed. And with each change the proportion of failures recorded became higher.

It is well known that the chances are small of finding a causative organism, whether bacillus or protozoon, in a stale stool. It was surprising to find that, once the stool was more than thirty minutes old, specimens one, two, and three hours after passage gave much the same proportion of positive results, and that it was only after three hours that the percentage again fell. The warmth and humidity of the air during the dysentery season, and the fact that material is usually sent for examination during the heat of the day, between 9 a.m. and 4 p.m., probably help the survival of dysentery bacilli in a mucous stool, and militate against it, by encouraging the growth of *B. coli*, in a faecal one.

Table I shows the percentages of positive results (*B. flexneri* or *B. shiga*) obtained from 150 consecutive cases showing a definite bacillary exudate, ranged according to age at the time of plating.

TABLE I

Age when plated	Percentage of total	Positive results
Less than half hour	15.9	93.7
Half to one hour	25.4	67.7
One to two hours	25.2	78.9
Two to three hours	20.0	73.3
Over three hours	13.5	40.0

From out-stations dealing with the laboratory, the following specimens were sent from every case of clinical dysentery: mucus in Churman and Teague's glycerine solution for culture, smears of mucus on slides for identification of the cellular exudate, smears on coverslips fixed in Schaudinn's solution for examination for protozoa. Most of these stations while no more than 12 to 24 hours distant by train, were from 3 to 7 days away by registered parcel post.

The percentages of successful isolations from definite bacillary exudates from local and out station cases were as under —

In 1926	Local	71 per cent
	Out-stations	46 „ „
In 1927	Local	56 „ „
	Out-station	43 „ „

### THE PRELIMINARY DIAGNOSIS

Our practice in reporting on cases was that of most laboratories nowadays that is, a preliminary diagnosis was made from the microscopical nature of the cellular exudate and was returned to the hospital by the man who brought the stool, or telegraphed in the case of out-stations, the object being that appropriate treatment should be begun as early as possible

We found, as have other workers that *shiga* infections were not necessarily severe, nor *flexner* infections mild, and medical officers did not gain by knowing the identity of the causative organism in bacillary cases, if proteins were excluded from the diet until the first named had been excluded. The question of whether to administer anti dysenteric serum was best decided on clinical grounds alone, and comparatively few cases required this treatment

### THE EXUDATE

During the two years under consideration the total numbers and the distributions of exudates, as classed by microscopical appearance, were as under —

Year	DEFINITE BACILLARY		INDEFINITE		PROTOZOAL	
	Number	Per cent	Number	Per cent	Number	Per cent
1926	140	69.7	41	20.4	20	9.9
1927	124	69.7	43	24.2	11	6.1

Throughout the report percentages for 1926 and 1927 are given separately rather than the average for the two years, since where they remain much the same it may be taken that the figures represent fairly accurately the usual relative annual incidence

The typical bacillary exudate is quite unmistakable under the microscope. It is worth recording that exactly the same exudate was seen in three cases of

typhoid fever which ended fatally without perforation, and in no case which recovered. We saw this exudate also in two or three cases of cholera amongst Indians, but it may be that this was merely the waking up of a chronic bacillary dysentery by the new infection.

As a rule, one glance down the microscope is sufficient to recognize the definite exudate, but not infrequently, especially where the patient has not reported sick at the onset of the disease, or in chronic bacillary cases, search has to be made before a patch of pus cells, macrophages, and ghost cells is met in the mass of mucus studded with occasional red blood cells.

An indefinite exudate, consisting largely of mucus, a few red cells, pus cells, cells from intestinal mucosa, and much cellular and unrecognizable granular debris, usually failed to produce any causative organism, either bacillus or protozoon. *Flexner* bacilli were isolated sufficiently frequently from such exudates to bear out the opinion that they are probably more often than not an indication of subacute, or chronic bacillary infection. This supposition is supported by the fact that one may see this picture rarely preceding, and more often following, a definite bacillary exudate. During the two years the latter was met a day or two after the former in fourteen cases, from seven of which *B. flexneri* was isolated and from one *B. shiga*.

Two adults and two children of one family, taken ill within forty-eight hours of each other, all showed indefinite exudates, and from three of these *B. morang* was isolated, while the fourth gave a negative result.

Pieces of mucus adhering to the faecal masses produced by an enema in a very constipated subject will often show the same elements as does the indefinite exudate, with the exception of the red blood cells.

In the absence of active pathogenic protozoa, there seems to be nothing one can call a definite protozoal exudate, but there are certain findings which encourage further search for amœbæ. The presence of a large number of eosinophiles, as pointed out by Anderson, is suggestive, as is agglutination of the red blood cells, as contrasted with the free-floating red cells in a bacillary exudate, and this latter characteristic alone, in an otherwise definite bacillary exudate, was considered sufficient evidence on which to base a tentative diagnosis of 'probable mixed infection'.

Charcot-Leyden crystals were present in fifteen acute dysenteries, and were accompanied by *E. histolytica*, either in the first specimen examined or during the following few days, in five of the patients, while a sixth showed this amœba in a relapse six months later. *Trichomonas hominis* in unusually large numbers was the only finding in three more cases, in two of which an occasional protozoon contained a red blood corpuscle. All the above nine patients were adults. Of the remaining six, five were children of ages ranging from 8 months to 4 or 5 years and in all these the exudate was definitely bacillary in character. No amœbæ were found in frequent examinations.

We never saw Charcot-Leiden crystals in a stool containing *E histolytica* cysts or in the absence of mucus and the conclusion suggested by these few cases is that their presence in an adult is very suspicious of active amœbic infection, but that in an infant they have not this significance. When they were met with in an adult it seemed justifiable to recommend a full course of treatment for amœbic dysentery.

The diagnosis of amœbic dysentery was never made on the finding of *E histolytica* cysts, as 10 to 15 per cent of Indians and a doubtful proportion of Europeans normally pass such cysts.

A not uncommon exudate amongst Indians was one definitely bacillary in character in cases which showed *E histolytica* a day or so later. Of six mixed infections diagnosed microscopically and by culture four began as definite bacillary cases, and the same sequence was seen in four out of eight amœbic dysenteries from which no pathogenic bacillus was isolated but which also were undoubtedly mixed infections. It seems quite clear that all these eight cases had a latent amœbiasis which was lit up by an acute bacillary infection without which the amœbæ would presumably have remained quiescent.

The reaction of the exudate in 201 consecutive local cases is shown in Table II. The term 'protozoal exudate' is here used only of one containing *E histolytica*.

TABLE II

Exudate	No	PERCENTAGE		
		Acid	Neutral	Alkaline
Bacillary	160	2.5	10.0	87.5
Indefinite	32	12.5	50.0	37.5
Protozoal	6	10.0		
Mixed	6	66.6		33.4

Acidity in a bacillary dysentery stool could usually be accounted for by the presence of urine.

In one mixed *B flexner* and *E histolytica* infection, the exudate was alkaline on the first day of illness, indicating a bacillary infection, and acid on the following day, when amœbæ were found in the stools.



The reaction of the mucus can be tested by any one with a book of litmus papers, and if, where facilities for microscopical and cultural examination are not available, dysentery cases with an acid reaction were treated as amœbic infections and all others as bacillary infections, the resulting errors of treatment would probably be fewer than at present

#### LABORATORY METHODS

Endo's medium was first used for plating, but was quickly abandoned in favour of litmus lactose agar, as giving better differentiation once reliable litmus, an absolute necessity, was obtained

An attempt was made to follow up the microscopical diagnosis referred to with a final diagnosis 24 hours later, by emulsifying suspicious colonies directly from the plate in a drop each of *shiga* and *flexneri* agglutinating sera. This proved useless as many *flexneri* strains were inagglutinable, and several other organisms, notably *B. pyocyaneus*, were frequently agglutinated by low dilution *flexneri* serum

Lactose, glucose, mannite, and dulcitol, phenol red milk, and peptone salt media were used for testing biochemical reactions. Many textbooks attribute to *B. flexneri* and other dysentery organisms quite definite action on these media, and it was hoped by using them to make at least a preliminary grouping, but it was soon evident that this was not possible. It may be permissible to remark that writers of reports and textbooks frequently omit to state the number of days organisms are incubated before readings are made, knowledge necessary to those who wish to use their results or repeat their work

Nearly all strains of *B. flexneri* isolated failed to produce any change in phenol red milk after the first slight acidity resulting from 24 hours' incubation. Their action on the sugar media could not be relied upon for identification, for many strains which were agglutinated by *flexneri* high titre serum gave varying degrees of acidity in lactose during the first seven days, and more rarely in dulcitol

Maltose, saccharose, dextrin, raffinose, arabinose inulin, sorbitol, and galactose were later added to the sugars already in use, and the action on these of many strains isolated were tabulated daily over a period of 14 days' incubation. Their effect on maltose and saccharose gave no definite help in identification, while that on the remaining sugars was so variable as to be useless as an aid to classification

As a result of testing fifty-seven strains of *B. flexneri* proved serologically, and watched in all the above media for fourteen days, this organism was defined for the purposes of this investigation as follows —A non-motile, Gram-negative, non-sporing bacillus producing acid without gas in glucose within 24 hours, a similar change in mannite which may sometimes be delayed for another 24 hours, and having as a rule no effect on either lactose or dulcitol. Most strains produced indol.

Table III shows the reactions on all media used of four strains of *B flexner* which were proved serologically and demonstrates the unreliability of their classification by biochemical action only

TABLE III

Flexner strains	Day	Lactose	Glucose	Mannite	Dulcic	Maltose	Dextrin	Raffinose	Arabinose	Adonite	Inulin	Galactose	Saccharose	Milk	Indol, 2nd day
Shaw	1st	As	✓	✓	—	✓	✓	✓	—	—	—	—	A	A	—
Smith I		As	✓	✓	—	✓	✓	As	—	—	—	—	—	A	+
Washington		—	✓	✓	—	✓	✓	—	—	—	—	—	A	✓	+
Jage Ram		—	A	✓	—	✓	✓	—	—	—	—	—	A	A	+
Shaw	5th	✓	✓	✓	—	✓	✓	✓	—	—	—	—	✓	A	
Smith I		As	✓	✓	A	✓	✓	—	—	—	—	—	—	✓	
Washington		As	✓	✓	—	✓	—	A	—	—	—	—	✓	A	
Jage Ram		—	A	✓	—	✓	A	—	—	—	—	—	✓	Alk	
Shaw	14th	A	✓	✓	—	✓	A	✓	—	—	—	—	✓	A	
Smith, I		As	A	A	✓	A	✓	—	—	—	—	—	—	A	
Washington		A	A	✓	—	✓	—	A	—	—	—	—	A	✓	
Jage Ram		—	A	✓	—	✓	A	—	—	—	—	—	A	Alk	

A = Acid

As = Slight acidity

## DYSENTERY AND OTHER ORGANISMS MET WITH

During 1926, one hundred and forty cases of dysentery in the acute stage showed a typical bacillary exudate, while only fifteen cases of amoebic dysentery were diagnosed

During 1927 the figures were one hundred and twenty-four and six respectively. The percentage representations are —

Year	Bacillary	Amoebic
1926	90.3	9.7
1927	95.4	4.6

From many of the bacillary cases no possible causative organism was isolated. If, for purposes of comparison, only those cases are considered to be of

bacillary origin from which either *B shiga* or *B flexner* was isolated the percentages are —

Year	Bacillary	Amœbic
1926 .	85 8	14 2
1927	89 5	10 5

There is no doubt then that, of acute dysenteries in the area investigated, bacillary dysentery is some nine times more common than amœbic

The organism most frequently isolated from bacillary infections was *B flexner*. The percentage frequencies of this and of *B shiga* were —

Year	<i>B flexner</i>	<i>B shiga</i>
1926	95 9	4 1
1927	94 1	5 9

During the two years, from two hundred and sixty-four cases showing definite bacillary exudates, either *B flexner* or *B shiga* was obtained from one hundred and twenty-four, or only 46·9 per cent. This is partly accounted for by the fact that a considerable number of specimens came through the post from stations several hundreds of miles away.

From purely local cases, these organisms were isolated from 71 per cent during 1926, from 56 per cent during 1927, the fall being undoubtedly largely due to the change in methods of bringing stools to the laboratory.

An organism resembling *B morgan* was obtained, in the acute, subacute, and chronic stages, from numbers of cases, but often in conjunction with *B flexner*. From acute stools it was isolated alone in 5—0 per cent of cases in 1926, in 11·3 per cent in 1927. It was not agglutinated by the patient's serum in the seven cases in which this test was made.

As already related, two adult and two infant members of one family were taken ill with mild dysenteric symptoms within forty-eight hours of each other. In all four the exudate was indefinite in character, and from three *B morgan* alone was isolated, while no possibly pathogenic organism was grown from the fourth case.

*B dysenteriae* Schmitz, as classified serologically, though it could not always be induced to produce indol, was recovered alone from 7·1 per cent of acute cases in 1926, from 4·0 per cent in 1927. It was agglutinated in 1—250 dilution of the serum of one out of four cases tested.

An organism resembling biochemically the members of the Salmonella group, but not agglutinated by any of the sera of that group, was occasionally seen in acute and more often in subacute and chronic dysenteries. It was frequently isolated in conjunction with *B flexner*, and we did not form the impression that it was a possible cause of dysentery. On the other hand, this organism was rarely met with in several thousand investigations of the fæces of healthy followers, and never in any other disease than dysentery. It was distinguishable from the

Salmonella group organisms by the immediate and intense production of alkali in phenol red milk

From two cases of typical acute bacillary dysentery occurring on the same day in the same unit and with no symptoms of food poisoning other than are common to the two diseases, *B. enteritidis* Newport was isolated

*B. enteritidis* Gartner was the only organism obtained from the stool of a patient with the symptoms of chronic dysentery, but was not necessarily the causative organism

*B. asiaticus* agglutinated by a serum made against a strain of this organism supplied by the R A M College, Millbank was isolated during the acute stage on three occasions

*B. dysenteriae* Sonne was in our experience very rare, as judged by serological tests, but several organisms which proved serologically to be strains of *B. flexner* gave the biochemical reactions of the former

The importance of sending stools to the laboratory for culture as early as possible in the disease is shown in Table IV, which gives the percentage of isolations of either *B. flexner* or *B. shiga* from 222 consecutive cases diagnosed bacillary dysentery on the appearance of the exudate

TABLE IV

Day of disease on which first specimen was received	Number of cases	Percentage of cases from which <i>B. flexner</i> or <i>B. shiga</i> was isolated
1st	104	67.1
2nd	50	44.0
3rd	26	47.7
4th	13	46.2
5th	6	50.0
6th	6	33.3
7th	5	40.0
After 7th	12	8.3

Both local and out-station cases are included in the above

It is obvious from this table that there is a much greater chance of isolating the infecting bacillus on the first day of disease than on any subsequent day

During the two years, organisms in the following proportions were isolated from the stools of dysentery cases in Mhow and the surrounding stations. Only those organisms which were present alone are included, if one case produced, for instance, both *flexner* and *morgan*, the former alone is counted. It is not suggested that all are pathogenic.

<i>B dysenteriae Flexner</i>	75.2	per cent
<i>B dysenteriae Shiga</i>	3.8	„ „
<i>B dysenteriae Schmitz</i>	9.5	„ „
<i>B dysenteriae Sonne</i>	1.3	„ „
<i>B æthycke Newport</i>	1.3	„ „
<i>B enteritidis Gærtner</i>	0.6	„ „
<i>B morgan</i>	7.0	„ „
<i>B asiaticus</i>	1.3	„ „

#### PERSISTENCE OF ORGANISMS IN THE STOOLS

For eighteen months out of the two years, no man left the hospital until his stools had given negative culture results for ten consecutive days. During this time we only recovered dysentery bacilli from one adult patient in the absence of visible mucus. This man had a persistent mild diarrhoea. The most careful examination, however, must be made before mucus is declared to be absent. On very many occasions, indeed, it was not seen until picked up by the copper wire spatula, and it is very doubtful whether it is safe to lay down that dysentery organisms are not being passed if 'visible mucus' is absent, since this term may easily be misunderstood by those responsible for the examination. It was not uncommon for a report of 'no mucus' to accompany a specimen, yet for this to be found in the laboratory, owing to the different methods of examination possible here and in the hospital.

During the remaining six months of the two years, patients were discharged from hospital when they had ceased to pass macroscopically visible mucus for ten days, each case being checked by two or three bacteriological examinations. The reason for this was that lactose fermenting bacilli of various kinds are frequently present in healthy fæces in this country, and the time necessary for the identification of such organisms isolated on, say, the tenth day leads to an unnecessary delay in the discharge of the patient.

As already stated, from one single *adult* case in two years a dysentery bacillus *B flexner* was recovered after all passage of mucus had ceased, but from him on many occasions during the thirty-five days between the onset of the disease and the final positive result. No mucus was seen after his fifth day in hospital. It is therefore very rarely that dysentery bacilli are recovered from adults when the presence of mucus is not demonstrable if a thorough search for it is made. But this is not true for children. *B flexner* was a common cause of 'green diarrhoea' with or without mucus in the stools, and when mucus was present the appearance

of the exudate was by no means always that of bacillary dysentery. Bile stained mucus contained some granular debris and a few pus cells and cells from the intestinal mucosa, and the picture was that so commonly seen in cases diagnosed 'colitis'. Red blood cells were exceptional.

The manifestation of a *flexner* infection in children was not confined to 'green diarrhoea,' typical bacillary dysentery being not uncommon.

In one hundred and twenty-seven *flexner* infections the exact date of onset was obtained, whether of the preliminary diarrhoea, or, where this did not open the illness, of the acute attack. The last day on which the organism was isolated from the faeces is shown in Table V. It will be seen that only 81.9 per cent were free of infection in ten days from the onset, leaving 18.1 per cent still carrying and 87.4 per cent were free in fourteen days, leaving 12.6 per cent of *flexner* cases passing dysentery bacilli for any period up to thirty-seven days from the onset of the illness.

TABLE V

Day of disease	<i>Flexner</i> CASES 127		<i>Shiga</i> CASES 6		<i>Schmitz</i> CASES 12	
	Cases	Per cent free of infection	Cases	Per cent free of infection	Cases	Per cent free of infection
2nd	31	24.4	5	83.3	6	50.0
3rd	29	47.2	1	100	2	66.6
4th	11	56.9				66.6
5th	9	62.9			1	75.0
6th	6	67.7			1	83.3
7th	7	73.2			1	92.5
10th	11	81.9			1	100
14th	7	87.4				
21st	9	94.5				
35th	6	99.2				
37th	1	100				

Six infections with *B shiga* and twelve with *B schmitz* are also included in the table. From the former the organism was not obtained after the third day of illness, from the latter after the tenth.

On such small numbers it is not justifiable to draw any certain conclusions, but the very marked contrast between the persistence of *B flexner* and of *B shiga* in the faeces suggests a reason not only for the difference between the numbers of *flexner* and *shiga* infections encountered in this as in other investigations in India, but also for the fact that *B shiga* is the cause either of sporadic cases or of sudden flaring epidemics, while *B flexner* dysentery goes on steadily all the year round with a natural rise in incidence in the fly season.

#### SEROLOGICAL INVESTIGATIONS

##### *Agglutinogenesis of local strains of B flexner in acute dysentery*

Some of the results of the serological investigation are difficult at present to interpret.

In order to determine the agglutinin response in bacillary dysentery, and to ascertain whether or not this is of any help in diagnosis, it was hoped to obtain blood serum from one hundred cases of acute dysentery, on admission to hospital, and on the 8th and 16th days after onset. It appears from the results obtained by Manifold and DeMonte that more information would have been gained by testing also one week later. A complete series of three tests was made in forty-five cases only, as the men ceased to volunteer blood for this purpose.

All the sera were put up against Oxford Standard emulsions of the five *flexner* types V, W, X, Z, and Y, Dreyer's technique was employed, and readings were taken after four and a half hours in the water bath. They were also tested against an Oxford Standard emulsion of *B shiga* and against laboratory emulsions of strains of *B sonne* and *B schmitz* received from the R. A. M. College, London. Results were recorded in standard agglutinin units, owing to the very varying degrees of agglutinability of the emulsions, with the exception of *B sonne* and *B schmitz* agglutinins, which are shown as percentages of full titre, as we had no standard emulsions of these organisms.

*B flexner* agglutinins were already present at the onset of the disease in 75.6 per cent of cases, showed a rise against one or more types in 64.5 per cent, showed a steady fall in 13.3 per cent, showed no change in 22.2 per cent.

One *E histolytica* infection only was included amongst these forty-five cases, and gave a rise of agglutinins, which was not unexpected, since it was believed to be a bacillary dysentery lighting up an old amoebiasis, though no causative bacillus was isolated.

*B flexner* was obtained from the stools of twenty-eight of the forty-five cases, and agglutinins against

<i>B flexner</i> V	were present in 15 or 53.6 per cent of these
<i>B flexner</i> W	„ „ „ 7 „ 21.3 „
<i>B flexner</i> X	„ „ „ 16 „ 57.1 „
<i>B flexner</i> Z	„ „ „ 7 „ 24.3 „
<i>B flexner</i> Y	„ „ „ 13 „ 46.4 „
<i>B shiga</i>	„ „ „ 2 „ 7.1 „
<i>B schmitz</i>	„ „ „ 3 „ 10.7 „
<i>B sonne</i>	„ „ „ 0 „ „

From none of the five cases which showed agglutinins against *B shiga* and *B schmitz* present in all five at the onset and remaining steady throughout the illness was either organism isolated.

The above results are interesting. Tables VI to XI (below) show the degree of agglutination of fifty-seven *flexner* strains by the different type sera, and in these it will be seen that only two were agglutinated by X serum and that most of the strains appeared to be of W type (Table VI). In Table XII it is seen that our stock W strain which came from a reliable source, does not give rise to X agglutinins in a rabbit, and yet more than half of the patients examined showed agglutination of X. Moreover, in one of these cases (Lovett) X agglutinins were higher than any other type throughout the illness, yet Lovett has been proved by absorption to be a W type. It appears certain, therefore, that the patient's serum does not respond specifically to the infecting type.

In four *B schmitz* infections, specific agglutinins were produced in one case only.

In two *B shiga* cases tested, specific agglutinins were produced in both.

#### *Agglutination of local strains of B flexner*

A large number of strains was inagglutinable, on first isolation, by *flexner* serum but most of them acquired agglutinability after subculture daily in broth for ten days, and as a routine procedure organisms were only tested after such subculture.

High titre agglutinating sera were prepared from rabbits for the V, W, X, Z and Y types of *B flexner*, using as antigens cultures supplied by the Department of Pathology and Bacteriology, Royal Army Medical College, London. Ten local strains were also used, but three did not serve as satisfactory antigens, and one was too toxic, and these were abandoned. These ten strains were chosen as representative of the local infection, and were from British and Indian cases both acute and chronic.

The six for which high titre sera were prepared were Allen, Shaw, Lovett, Jage Ram, Bahadur Shab and Ram Harak. All organisms investigated were tested against these and the V, W, X, Z and Y sera, and many against *shiga*, *sonne* and *schmitz* high titre sera, because some during early or late incubation had given the biochemical reactions of these organisms.



Fifty-seven strains of *B flexneri* were tested, and none were agglutinated by any of the last three sera

Tables VI to XII show the results of the agglutination tests. The figures represent the percentage of the full titre of the serum to which each organism was agglutinated, and should theoretically show the type to which the strain belongs. Percentages have been raised or lowered to the nearest 5, in order to simplify the tables.

Twenty strains (Table VI) are agglutinated in highest percentage by *W* serum and should therefore be of type *W*. As yet absorption tests have not completed the proof of this.

Strains 8 and 22 are, on this showing, type *Y*, or closely related thereto, but absorption tests have proved them both to be type *W*.

Table VII consists of three strains which must provisionally be considered to be of type *V*.

The strains in Tables VIII and IX are as yet unclassified. Strain 32 resembles strains 8 and 22, but is serologically related to 'Shaw,' which these are not.

In Table X is the only organism yet tested which appears to be of type *X*.

Table X is the most interesting, and it is amongst the strains therein that a type of *B flexneri* of different antigenic structure from those determined by Andrews and Inman may be present, though we have not yet obtained complete proof of this. Until recently, none were agglutinated, in spite of frequent subculture, by any of the high titre sera prepared for the known *flexneri* types, but the 8 hours agglutination method advocated by Manifold and DeMonte has not yet been tried.

All except the last seven were agglutinated by one or more of the local sera, so that they are presumably *flexneri* strains, and both Shaw (40) and Ram Harak (41) when tested very recently showed agglutinability by the type sera, and should properly be removed into other groups, but are retained in their present group for the purposes of this paper because they remained magglutinable by type sera for nearly two years. It is therefore not impossible that strains 51 to 57 may in time acquire demonstrable serological relationship with the known *flexneri* types.

As judged by their agglutinability, the fifty-seven strains investigated were divided up as follows —

<i>B dysenteriae Flexneri W</i>	38.6 per cent
<i>B dysenteriae Flexneri V</i>	5.3 „
<i>B dysenteriae Flexneri X</i>	1.8 „
As yet unclassified	54.3 „

It appears, however, that the method of classification used by Andrews and Inman is not in all cases reliable. This has been shown above in the case of Lovett (No. 8). Table XIII gives the result of testing four strains three times, with about twelve months' interval between each test. Other strains similarly tested showed fixed or increased agglutinability, but in these four quite marked changes have taken place. The sera used were usually of 1—5,000 titre or over, no serum was employed which had not a titre of 1—1,000.

*Allen* was agglutinated on isolation, after the routine ten subcultures, by H serum but only to twenty per cent of its full titre. It was also agglutinated to full titre by *Jagu Ram* and *Bahadur Shah* sera. A few weeks later it had lost all agglutinability except by its own serum and the titre of the latter had fallen very considerably from 1—1,250 to 1—250 the change being probably wholly in the organism and not in the quality of the serum.

This state was retained until quite recently, when it was found to have regained slight agglutinability by *Bahadur Shah* serum. *Jagu Ram* serum and subculture became contaminated and were lost.

The strain *Bahadur Shah* has shown small changes the chief one being the loss of agglutinability by *Allen* serum.

*Lovett*, while becoming generally more highly agglutinable is not now touched by *Shaw* serum, but has acquired agglutinability by *Bahadur Shah* serum.

*Shaw* is an organism which for over a year deposited heavily in broth in twenty-four hours leaving the supernatant fluid quite clear a characteristic which was only noticed in a few strains one of them being *Ram Haral* (No. 11), obviously closely related to *Shaw*. The latter was at first agglutinated to a certain degree by Z serum, and very slightly by I and Y sera but later lost all agglutinability except by the homologous serum and by *Ram Haral*. That this was not the same general loss of agglutinability as was shown by *Allen* is proved by the fact that *Shaw* serum had throughout a titre of 1—5,000, which has only fallen since the second test noted in the table. *Shaw* then gradually ceased to deposit, until now it gives a uniform suspension in broth and this change has been accompanied by the growth of agglutinability by W serum.

Absorption tests have not yet revealed the true nature of *Shaw*, but it is obvious that in the case of this organism, as with *Lovett*, the 'percentage titre' method of classification fails to place it in its type. *Lovett* by this method is of the Y type, but is proved by absorption to be a W type. *Shaw* would at first have been labelled a Z type, and now a W type.

Some of the results obtained in the serological investigation of these strains might be partly explained on the assumption that there are group and specific phases of *B. flexner*. Or it may be that at least some *flexner* strains are convertible, under circumstances not determined, into any or all of the different types, that some portions of the mosaic of antigens may be latent and capable of stimulation.

A *flexner* strain was in three instances isolated from two members of the same family, and the organisms of each pair were so closely related, as judged by agglutination tests, as to suggest that they are the same organism in different stages of agglutinability. Strain 27 (Connell, I) is agglutinated by *Allen* serum, strain 6 (Connell, II) is not, otherwise they are identical. Strain 20 (*Hendricks*, I) as strain 49 (*Hendricks*, II) become more widely agglutinable, and the same may be said of strains 5 and 25 (*Lockett*, I and II).

One fact has been noted in preparing high titre agglutinating sera against locally isolated strains of *B. flexner*. Their titre falls more rapidly than does that of

sera made against type strains procured from the R A M College, London, which have been subcultures in laboratories for some years. If the agglutino-genetic powers of an organism are a measure of the protective bodies it is capable of stimulating when employed as an antigen, it appears that a well-established laboratory strain will be more suitable for inclusion in a prophylactic vaccine than will a strain recently isolated.

#### AN ORAL VACCINE

The vaccine as finally decided upon was an emulsion containing *B flexner V* and *W*, and the four local strains *Allen*, *Bahadur Shah*, *Lovett*, and *Shaw* in equal quantities. It was made by washing off agar in saline, killing at 70°C, and preserving in 30 per cent sterilized glycerine.

The vaccine was given in two doses of one ounce each, each dose containing 90,000 million organisms in all, or 15,000 million of each strain, and was taken in the early morning two hours before breakfast, the only nutriment allowed being a cup of tea, with the idea that this would wash the emulsion more quickly through the stomach.

This method was chosen as the result of animal experiments in which it gave the greatest protection.

No reaction was ever complained of by any of the one thousand volunteers who have received the vaccine, beyond slight discomfort experienced by a very few. This was described as a feeling as of very mild colic which began within an hour of swallowing the first dose, quickly passed off, and was not followed by diarrhoea. In two cases only was there any severe reaction, two volunteers being seized with violent colic and purging for some hours after their first dose. They had attended a regimental guest night on the previous evening, from which it may be argued that alcohol should be partaken of only sparingly on the night before the vaccine is taken. In neither case was there any reaction after the second dose.

Children from the age of four years upwards have received appropriate doses with the same absence of reaction.

The disadvantage of this vaccine is the length of time it takes to make sufficient for any large quantity of men, and this is especially felt in a laboratory which has to deal with the routine work of several hospitals. It will be noticed that the number of organisms in two doses of vaccine is nearly fifty times greater than that in the 15 c c of T A B vaccine which constitute the usual two anti-typhoid inoculations.

The cost is much smaller than that of Besredka's bilivaccin, being only 3 19 annas for the two doses, as compared with Re 1, but this is the price of media, kerosene for autoclaving, etc., only, and a method of cost-accounting would undoubtedly find it to be considerably higher than this sum.

It was unfortunately not possible to try the vaccine on any appreciable number of men until July 1927, when the dysentery season was in full swing, and then only 128 men could be given it.

This number of course is much too small a one on which to base any conclusions, but it is worth recording that the results to date in the unit from which these volunteers came are as follows —

Admission to hospital	NON VACCINATED		VACCINATED	
	Number	Per cent	Number	Per cent
Bacillary dysentery	24	3.27	1	0.75
Diarrhoea	13	1.77	0	
TOTAL	37	5.04	1	0.75

Over one thousand volunteers, British and Indian, have received this oral vaccine since July 1927 but sufficient time has not elapsed to allow one to judge whether any immunity has followed except in the unit quoted, and there the occurrence of one or two cases amongst the small number of vaccinated would completely upset the figures.

It was pointed out by the Officer Commanding this unit that the human element introduces what is probably a very real source of error into the consideration of results of preventive treatment where the treated are volunteers, especially so when as here, the immediate effects of the treatment are in considerable doubt. The men who volunteer in such a case are the cheerful, healthy type, who would probably be less likely than others to succumb to infection, and are therefore likely to give a false picture of success to a vaccine which may have given them no protection whatever.

In the animal experiments, we did not find that feeding rabbits with large doses of an emulsion of either *B flexneri* or *B shiga* was followed by the appearance of agglutinins in the blood.

A further report on this oral vaccine will follow.

#### SUMMARY

1 Over ninety per cent of dysenteries in the area examined were bacillary infections, and in ninety per cent of these the organism responsible was *B dysenteriae flexneri*. Less than ten per cent were caused by *E histolytica*.

2 Over ninety per cent of positive cultures can be obtained from bacillary dysenteries if the stool is plated within half an hour of passage. After the stool was more than three hours old, only forty per cent of successful cultures was obtained.

3 *B flexneri* appears to persist in the stools much longer than does *B shiga* in efficiently treated cases.

4 Fewer errors of diagnosis would be made if cases in which the mucus was acid were titrated as amoebic dysenteries, and all others as bacillary dysenteries. This is a suitable test for practitioners who have not access to a microscope or a laboratory.

5 'Green diarrhoea' in children is frequently caused by *B flexner*.

6 Many amoebic dysenteries are probably cases of chronic amoebiasis lit up by an infection with a dysentery bacillus.

7 Charcot-Leyden crystals are not indicative of amoebic infection in infants.

8 The agglutinins produced during an attack of *flexner* dysentery are not specific to the type of the infecting organism.

9 *B flexner* W appeared to be the predominant type in the locality but the 'percentage titre' method of typing does not always lead to a correct classification.

10 An oral vaccine, consisting of an emulsion of different types of *B flexner* in saline and glycerine, and administered in a total dosage of 180 000 million organisms, has given satisfactory results in an extremely limited trial. It failed to produce agglutinins in experimental rabbits, as did a similar trial with a *B shiga* emulsion.

We wish to express our very real appreciation of the willingness with which a large number of the staff of several military hospitals helped us in this investigation in supplying details of cases and in ensuring that all material reached the hospital as fresh as the means at their command would allow.

TABLE VI

No	Organism	S E R A														
		Patient	V	W	X	Z	Y	Shiga	Sonne	Schmutz	Jage Ram	Lovett	Shaw	Allen	Bahadur Shah	Ram Harak
1	Coulson	0	25	50	0	0	10		0		60	40	100	100	25	10
2	Dent		25	30	0	0	25		0		40	40	10	20	20	10
3	Balderston	0	10	20	0	0	15		0		0	30	20	10	0	30
4	Elliott		25	30	0	0	30	0	0		0	25	10	0	0	10
5	Lockett, I		5	10	0	0	10		0		0	30	20	0	0	25

TABLE VI—contd

		S E R A														
No	Organism	Patent	1	2	3	4	5	Shiga	Sonne	Schmitz	Jago Ram	Lovett	Shaw	Allen	Bahadur Shah	Ram Harak
6	Connell, II		10	20	0	0	5		0		10	15	40	0	0	0
7	Mainev	1 50	5	10	0	0	5		0		0	30	25	0	0	0
8	Lovett	0	20	50	0	0	100				0	100	0	0	40	100
9	Jage Ram	0	0	75	0	0	0				100	0	0	90	30	0
10	Gopal Singh	1/25	0	30	0	0	5	0	0	0	20	0	0	30	25	0
11	Jumma	1 50	0	10	0	0	10	0	0	0	20	0	0	10	30	0
12	Long	0	0	10	0	0	10		0		30	0	0	30	20	0
13	Allen	0	0	20	0	0	0		0		100	0	0	100	100	0
14	Bahadur Shah	0	0	80	0	0	0	0	0		100	0	0	80	100	0
15	Borzier		0	80	0	0	0				40	0	0	40	20	0
16	Cook, I		0	20	0	0	0		0		25	0	0	30	30	0
17	Hawkins	0	0	20	0	0	0		0		10	0	0	20	20	0
18	Hildrick Smith		0	30	0	0	0		0		20	0	0	20	80	0
19	Rudman	0	0	10	0	0	0		0		50	0	0	25	10	0
20	Hendricks, II		0	10	0	0	0		0		0	0	0	10	10	0
21	Cooke, II		0	40	5	0	15		0		10	30	20	0	0	20
22	Morley		20	20	0	0	80				0	100	0	0	50	0

TABLE VII

No	Organism	S E R A														
		Patient	V	W	X	Z	Y	Shiga	Sonne	Schmitz	Jage Ram	Lovett	Shaw	Allen	Bahadur	Ram Harak
23	Pick		30	10	0	0	10		0		0	30	15	0	0	5
24	Gadgill		30	0	0	0	10		0		0	10	15	0	0	0
25	Lockett, II		25	0	0	0	0		0		0	10	5	0	0	5

TABLE VIII

26	Taylor, III	0	5	0	0	0	5		0		0	10	5	0	0	0
27	Connell, I		10	10	0	0	10		0		10	30	30	10	0	5
28	Garbutt	0	10	10	0	0	10		0		0	20	25	0	0	0
29	Rhodes, James		10	10	0	0	0				0	10	10	0	0	0
30	Ghulam Mohd	0	5	0	5	5	0		0		0	0	0	0	0	10
31	Heath	0	5	0	5	5	0		0		5	5	10	20	10	0

TABLE IX

32	Caley	0	0	0	5	0	20		0		10	30	20	0	0	20
33	Taylor, I	0	0	0	0	5	0				0	2	0	0	100	0
34	Mohd Hayat	0	0	0	0	0	5		0		5	5	10	0	5	5
35	Ivatts		0	0	0	0	5				5	10	10	0	0	25
36	Hari	1/25	0	5	0	10	0		0		0	0	0	0	0	10

TABLE X

No	Organism	S E R A														
		Patient	V	W	X	Z	Y	Shiga	Sonne	Schmitz	Jage Ram	Lovett	Shaw	Allen	Bahadur Shah	Ram Harak
37	Smith I		20	25	80	0	0				5	50	0	0	0	0

TABLE XI

38	Ranga		0	0	0	0	0			0	50	50	100	200	100
39	Ram Singh		0	0	0	0	0			0	0	20	100	100	0
40	Shair	0	0	0	0	0	0			0	0	100	0	0	100
41	Ram Harak	0	0	0	0	0	0			0	0	100	0	0	100
42	Riffer		0	0	0	0	0	0		0	1	0	0	100	0
43	Smith, II	0	0	0	0	0	0			20	0	0	0	0	0
44	Bakshi Khan	0	0	0	0	0	0	0		100	0	0	70	100	0
45	Horner, II	0	0	0	0	0	0	0		10	0	0	5	0	0
46	Roughton	0	0	0	0	0	0	0		5	0	0	0	0	0
47	Hanson	0	0	0	0	0	0	0		0	0	0	25	0	0
48	Ruddock	0	0	0	0	0	0	0		0	0	0	5	0	0
49	Hendricks, II		0	0	0	0	0	0		0	0	0	20	0	0
50	Brownsea		0	0	0	0	0			0	2	0	0	0	0
51	Priest		0	0	0	0	0	0		0	0	0	0	0	0
52	Plumb	1/25	0	0	0	0	0	0		0	0	0	0	0	0
53	Clavin	0	0	0	0	0	0	0		0	0	0	0	0	0
54	Davis	0	0	0	0	0	0	0		0	0	0	0	0	0
55	East	1/25	0	0	0	0	0	0		0	0	0	0	0	0
56	Hanscowan	0	0	0	0	0	0	0	0		0	0	0	0	0
57	Tulleston		0	0	0	0	0	0		0	0	0	0	0	0



TABLE XII

No	Organism	S E R A										
		V	W	X	Z	Y	Jage Ram	Lovett	Shaw	Allen	Bahadur Shah	Ram Harak
	Flexner V	100	20	10	0	50	5	10	20	0	30	5
	„ W	10	100	0	0	25	0	100	0	0	23	0
	„ X	20	0	100	40	40	5	10	20	0	80	3
	„ Z	10	0	0	100	0	15	100	40	0	80	3
	„ Y	20	30	50	80	100	0	10	20	0	10	1

TABLE XIII

Strain												
Allen	0	20	0	0	0	100	0	0	100	100	0	
	0	0	0	0	0	0	0	0	100	0	0	
	0	0	0	0	0		0	0	100	20	0	
Bahadur Shah	0	80	0	0	0	100	0	0	80	100	0	
	0	0	0	0	0	100	0	0	0	100	0	
	5	10	0	0	5		5	0	0	100	0	
Lovett	5	5	0	0	5	0	100	30	0	0	0	
	20	50	0	0	100	0	100	0	0	40	100	
	25	60	0	0	100		100	0	0	30	100	
Shaw	5	0	0	20	5	50	0	100				
	0	0	0	0	0	0	0	100	0	0	100	
	0	15	0	0	5		0	100	0	0	50	

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- MEDICAL RESEARCH COUNCIL Special Report Series, No 42

# THE COMPOSITION OF URINARY CALCULI

BY

MAJOR CLIVE NEWCOMB D.M.F.C., I.M.S.,

WITH THE TECHNICAL ASSISTANCE OF

S. RANGANATHAN, B.A., A.I.I.Sc.,

*From the Nutritional Research Laboratories, I.R.F.A., Pasteur Institute,  
Coonoor, S. India*

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IN a previous report(1) in connection with the investigation into stone-in-the-bladder in progress in these laboratories the results of the analyses of the first 100 of the human stones collected were given. The remaining stones (an additional 126) have now been analysed and are reported in this paper. Of the 226 stones, 221 were vesical and 5 renal. The latter form a separate class and a short note on their composition is included. The 221 vesical stones form an extensive series from all parts of India and afford material for a detailed discussion of the general composition of such stones in India. With each of the stones a medical-dietetic history of the case has been received but the discussion of the relationship of such factors as age, sex, diet, etc., to the composition of the stone is reserved for a subsequent paper.

## METHODS OF ANALYSIS

For the large stones (18 in addition to the 100 previously reported) the method of analysis used was that described in the previous report. The results for each stone are given in Table I.

The remaining stones (73) were too small for this method to be used conveniently and a micro-scale method was worked out and has been described in detail in a separate paper(2). For the small stones this micro-method was used and the results for each stone are given in Table II.

It will be noticed that in these smaller stones there are on the average more oxalates and correspondingly less nitrogen.

TABLE I

*Showing the results of analysis*

1	2	3	4	5	6	7	8	9
Stone number	Moisture as per cent of original stone	ASH		AS PERCENTAGES OF THE DRY STONE				
		Total	Insoluble	Total nitrogen	P <sub>2</sub> O <sub>5</sub>	CaO	MgO	CO <sub>2</sub>
9	20.7	40.7	2.45	15.8	23.5	9.4	4.3	3.6
14	18.0	80.6		2.24	41.0	35.4	2.6	0.7
23	18.2	24.6	Trace	25.7	17.6	0.91	5.6	0
24	6.3	14.8		27.8	7.9	6.6	+	0
27	4.85	15.8		19.4	0	15.0	+?	18.9
30	2.05	3.5		30.2	0	2.97	0	3.0
31	9.6	4.3		29.6	0	4.1	0	5.0
32	3.3	5.4		28.5	0	5.1	+	5.7
36	8.8	36.3		11.0	11.2	24.6	+	22.1
40	2.34	15.3		19.7	+	14.6	+	18.3
51	21.6	47.4	Trace	12.4	21.3	15.8	10.0	8.6
61	3.4	38.0		0.7	+	36.0	+	44.5
63	3.25	24.0	Trace	15.3	1.52	20.3	+	25.6
69	6.2	29.5		15.8	7.3	19.5	1.6	17.7
71	5.3	33.2		6.66	2.0	29.5	0.84	39.5
72	10.9	35.1		12.1	8.6	21.9	3.6	21.9
73	2.8	38.0		0.51	+	37.0	+	45.5
76	3.9	7.8		27.8	+	6.6	+	8.1
81	2.9	7.3		29.5	+	6.6	+	7.5
88	4.25	5.6		31.5	+	2.4	+	0
89	3.6	8.64		30.8	1.22	5.5	+	3.8
93	5.5	23.6	Trace	20.8	6.3	15.2	+	11.7
98	3.15	5.1		33.5	0.9	1.4	2.5	0

TABLE I—concl'd

1	2	3	4	5	6	7	8	9
Stone number	Moisture as per cent of original stone	ASH		AS PERCENTAGES OF THE DRY STONE				
		Total	Insoluble	Total nitrogen	P <sub>2</sub> O <sub>5</sub>	CaO	MgO	CO <sub>2</sub>
99	8.2	33.3		16.3	10.9	19.8	1.56	12.9
106	15.0	56.0	2.8	5.7	19.7	26.6	5.9	17.6
107	29.4	68.1	Trace	6.4	35.3	17.9	14.5	2.17
116	34.7	67.1		9.1	35.3	8.8	19.0	+
129	5.8	15.7		28.5	6.0	5.8	2.1	2.23
132	1.8	10.7		24.9	+	9.2	+	10.7
139	9.6	18.6		21.8	6.9	7.9	3.7	7.5
143	15.4	40.0		9.5	11.3	20.7	5.0	24.0
150	36.4	61.0	Trace	11.2	37.6	1.8	22.6	0
160	3.2	32.7		5.0	+	30.8	+	37.9
168	15.8	65.7		7.9	29.3	27.2	6.6	1.3
182	1.6	3.1		30.1	0	2.86	+	4.05
183	44.0	79.2		4.5	46.6	0.6	29.0	+
184	1.4	0.56		32.7	0	0.5	+	0
185	42.1	74.0		5.1	46.1	1.5	26.0	0
188	1.85	0.5		32.6	0	0.3	+	0
189	21.5	77.0		1.75	35.0	32.8	8.2	4.94
191	17.2	53.3		12.8	27.9	18.3	6.7	1.0
194	3.9	44.8		1.24	7.9	35.7	+	37.9
193	3.7	24.3		14.0	+	22.0	+	27.5
195	2.9	3.24		30.6	0	2.5	+	2.9
196	10.4	27.4	Trace	18.1	7.5	15.1	3.4	16.3
197	5.1	44.4		3.7	9.3	33.8	+	32.1
198	4.4	10.9		30.0	3.9	6.2	+	2.7
202	2.0	11.7		22.1	+	11.0	+	14.2

TABLE II—*contd*

1	2	3	4	5	6	7
Stone number	Moisture as per cent of original stone	Total nitrogen	AS PERCENTAGES OF THE DRY STONE			
			P <sub>2</sub> O <sub>5</sub>	CaO	MgO	C <sub>2</sub> O <sub>3</sub>
206	1.57	29.8	0.39	4.2	0	4.9
207	2.02	32.3	1.36	6.0	0	5.14
208	2.86	19.2	2.55	14.9	0	19.4
209	3.22	4.72	2.73	31.0	0	25.7
210	14.0	20.6	6.17	12.7	+	5.5
211	6.3	10.0	3.73	24.7	+	32.2
212	3.15	1.44	0.88	34.2	0	40.0
213	2.25	17.1	0.46	18.0	0	22.2
214	3.48	22.9	1.53	11.5	0	13.2
215	2.98	2.24	0.68	33.5	0	39.7
216	3.43	1.07	1.02	35.1	0	42.8
217	10.7	29.1	0.46	6.3	0	7.8
218	4.6	1.65	4.9	33.8	0	35.0
219	7.15	8.4	5.12	25.4	+	30.2
220	1.42	27.6	0.40	5.67	0	7.0
221	1.8	27.0	1.96	6.5	0	6.35
222	2.8	21.7	4.5]	12.64	0	10.7

TABLE II—*concl'd*

1	2	3	4	5	6	7
Stone number	Moisture as per cent of original stone	Total nitrogen	AS PERCENTAGES OF THE DRY STONE			
			P O <sub>2</sub>	CaO	MgO	C <sub>2</sub> O <sub>3</sub>
223	1.39	31.3	+	3.7	0	5.5
224	4.13	13.1	4.22	23.8	0	27.4
225	1.63	31.3	+	3.68	0	4.9
226	3.54	27.2	+	5.85	0	7.45
227	3.98	30.0	+	1.43	0	2.74

## THE GENERAL COMPOSITION OF THE STONES

The average composition of the 221 vesical stones is shown in Table III

TABLE III

*Showing the mean composition of the 221 vesical stones*

Constituent	Mean per cent	Standard deviation	Median per cent	} as percentages of the dry stone
Moisture	7.2	8.7	3.7	
N	17.9	11.0	19.7	
P O <sub>2</sub>	6.9	10.8	3.3	
CaO	14.0	11.9	11.4	
MgO	2.0	3.1	indeterminate	
C O <sub>3</sub>	14.0	13.2	8.6	

The distributions of the various constituents of the stones are shown in diagrams 1 to 6. These distributions are very asymmetrical and for the most part approach a J-shape. In consequence a large error is made if the means are calculated from grouped values unless the grouping is made very fine. The one exception to a more or less J-shaped distribution is the total nitrogen which is definitely U-shaped. This means that stones tend to have either a lot of or a little nitrogen and stones with an average amount are less common. In other words stones tend either to be urate or non-urate—though the majority of the stones have some uric acid or urates in them.

DIAGRAM 1

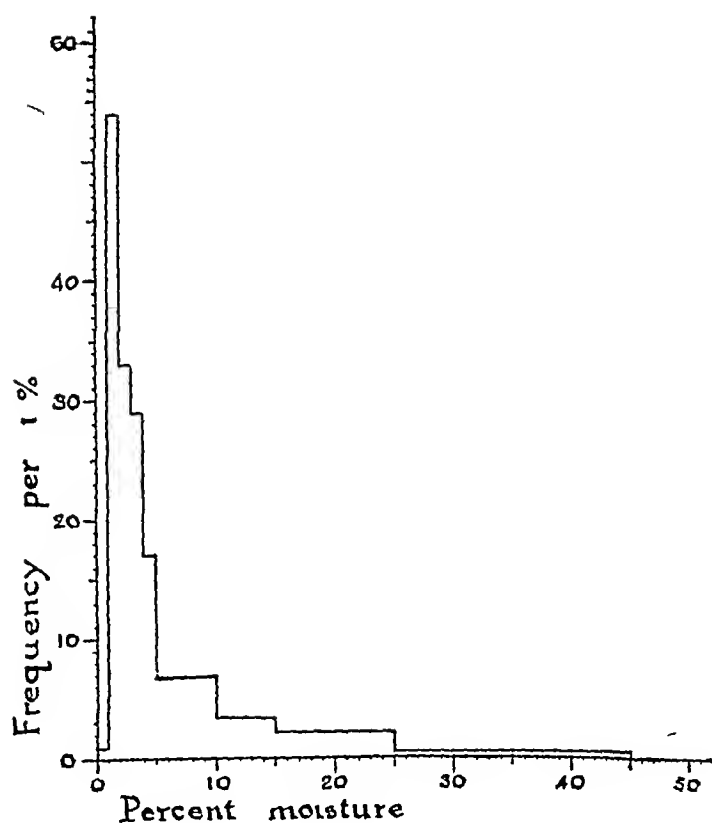
*Distribution of moisture.*

DIAGRAM 2

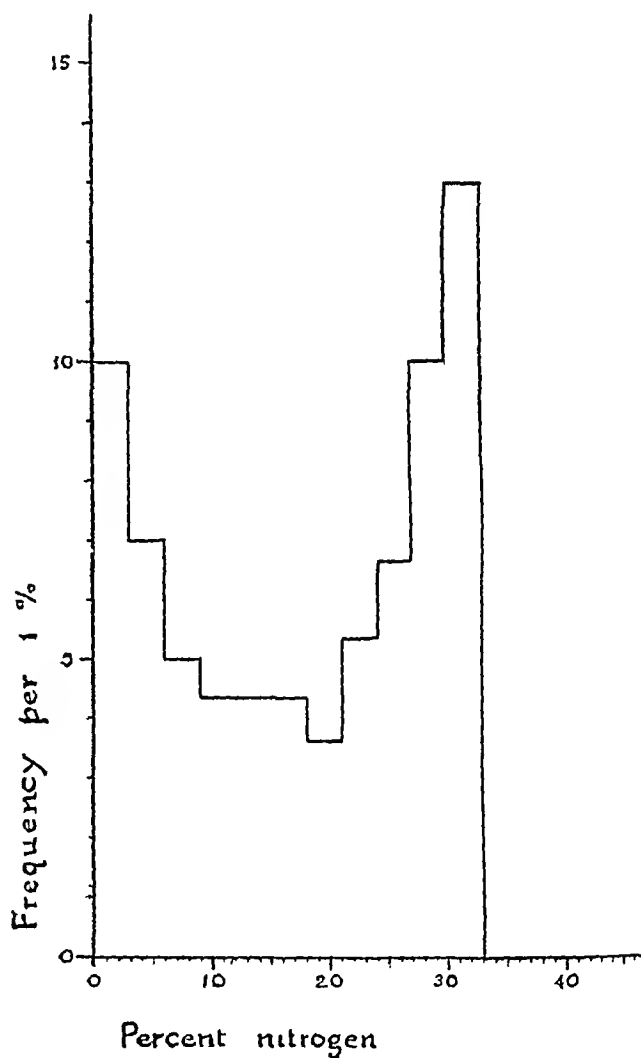


DIAGRAM 3

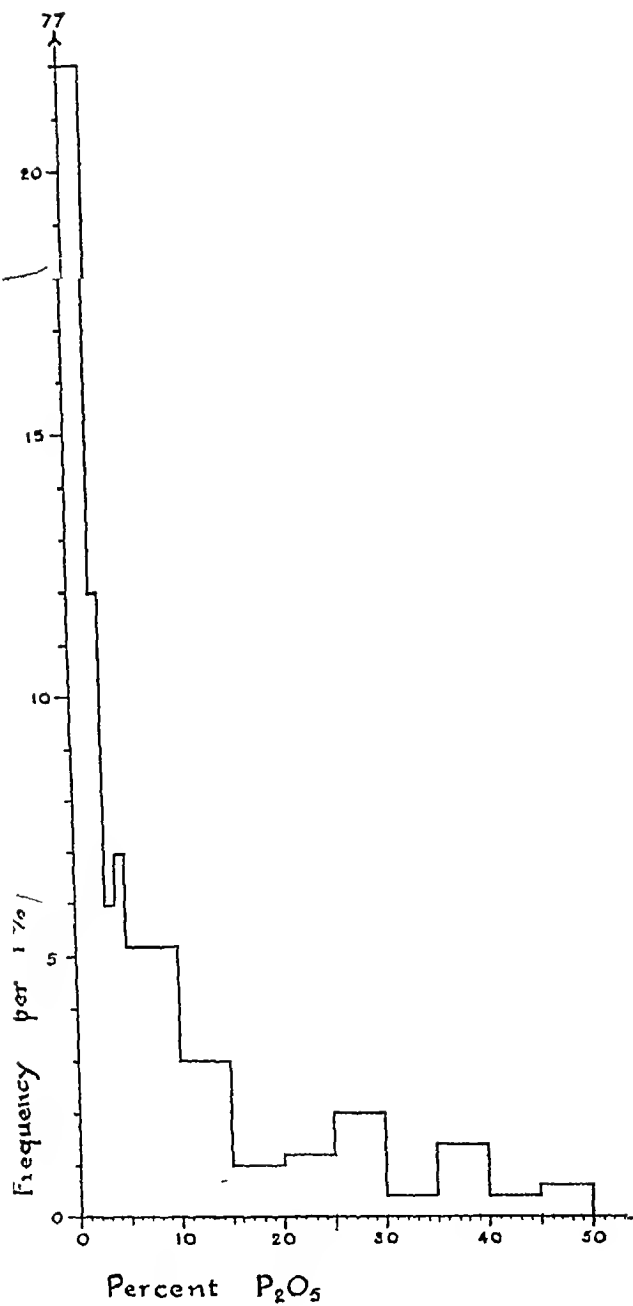


DIAGRAM 4.

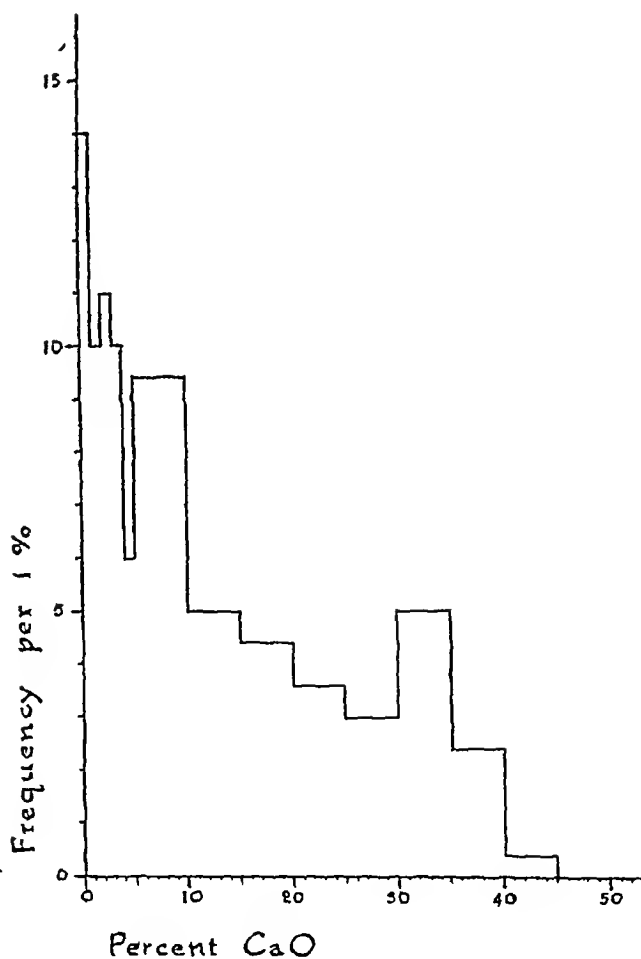




DIAGRAM 5

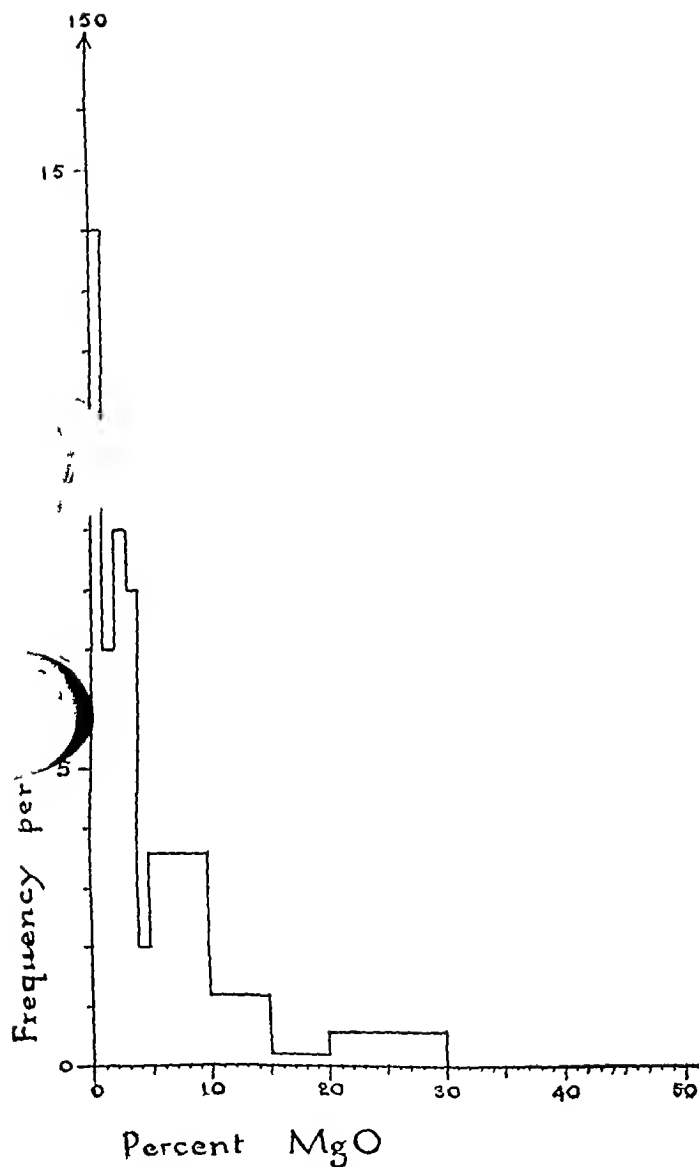
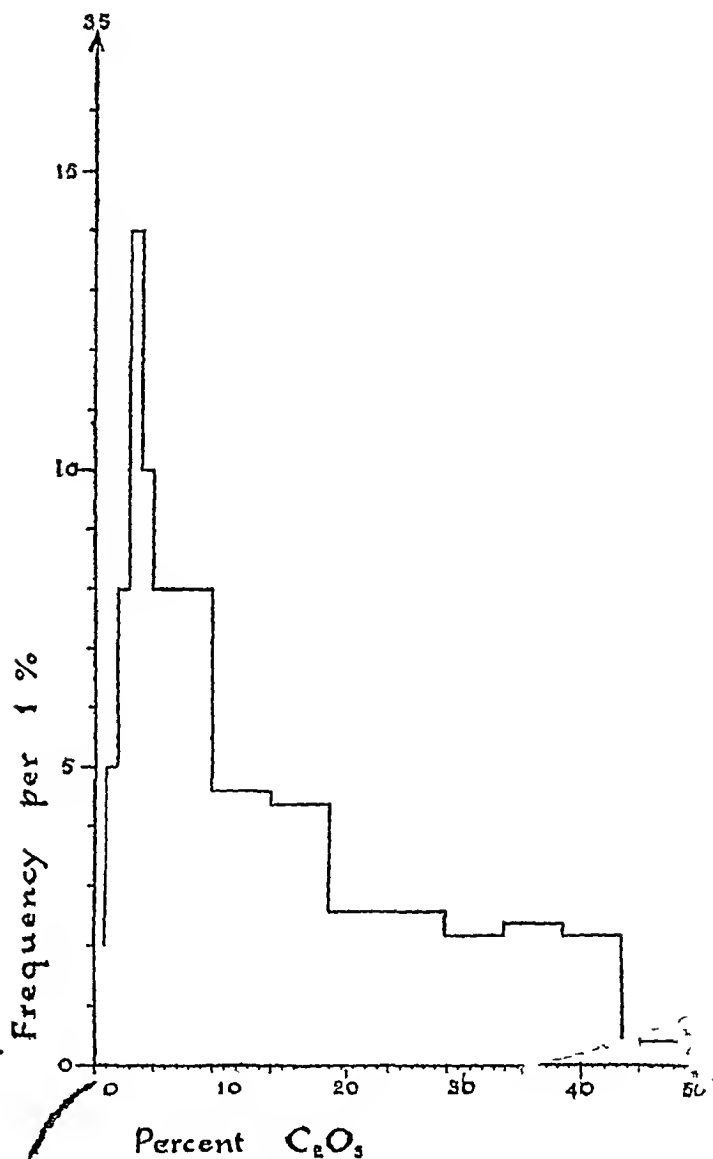


DIAGRAM 6



The phosphate distribution shows two rises one between 25—30 per cent  $P_2O_5$  and the other between 35—40 per cent. The first of these is due presumably to  $Ca_3(PO_4)_2$  since it corresponds to the very marked rise in the CaO curve between 30 and 35 per cent, the second is probably due to magnesium ammonium phosphate and corresponds to the rise in the MgO curve between 20 and 30 per cent.

The nature of the compounds, into which such constituents as have been determined are combined, is indicated by the associations between the constituents. Using the capital letters W, N, P, C, M and O to mean the stones containing more than the mean amounts of water, nitrogen, phosphate, calcium, magnesium and oxalate, the associations between these constituents are shown in Table IV.

TABLE IV

*Associations between constituents in 221 vesical stones*    *All positive classes*  
*Classed from means*

[illegible]

## Association coefficients of constituents of 221 vesical stones

	By grouping from means	By grouping from medians
W N	-0.72	-0.71
W P	+0.97	+0.93
W C	+0.35	+0.51
W M	+0.99	
W O	-0.19	+0.08
N P	-0.84	-0.80
N C	-0.98	-0.95
N M	-0.64	
N O	-0.87	-0.81
P C	+0.50	+0.69
P M	+0.98	
P O	-0.08	+0.29
C M	+0.16	
C O	+0.95	+0.94
M O	-0.38	

The following deductions may be made from these associations —

1 Stones with a large amount of phosphate have a large amount of moisture in them and urate stones have but little

2 Nitrogen is negatively associated with all the other constituents, that is to say for the most part the nitrogen exists in some form uncombined with calcium, magnesium, etc., and presumably as uric acid. The negative associations of the nitrogen with magnesium (NM—0.64) and to a lesser extent with phosphate (NP—0.84) are less marked than with calcium (NC—0.98), indicating that in stones which contain magnesium this is present in combination with nitrogen and phosphates presumably as magnesium ammonium phosphate (*vs*).

3 The association of oxalates with calcium is very marked (CO = +0.95) and *per contra* the association of oxalates with the other constituents is negative or insignificant, meaning that the oxalates exist as calcium oxalate.

4 The magnesium is markedly associated with phosphates (PM = +0.98) and less negatively associated with nitrogen than the other constituents, indicating that the magnesium exists as magnesium ammonium phosphate (*vs*).

These conclusions are confirmed by the relations between the constituents in the average stone (*v* Table III). If the 14.0 per cent of  $\text{C}_2\text{O}_3$  is calcium oxalate it will be united with 10.9 per cent of the  $\text{CaO}$ , leaving 4.0 per cent. If this 4.0 per cent  $\text{CaO}$  is united with  $\text{P}_2\text{O}_5$  to form  $\text{Ca}_3(\text{PO}_4)_2$  it will account for 3.38 per cent  $\text{P}_2\text{O}_5$ . If the 2 per cent of  $\text{MgO}$  is in the form of  $\text{Mg NH}_4 \text{PO}_4$  it will account for 3.55 per cent  $\text{P}_2\text{O}_5$ . The actual percentage of  $\text{P}_2\text{O}_5$  is 6.9 and  $3.38 + 3.55 = 6.93$  so that the whole of the phosphate is accounted for. The above calculation is only true for the average stone. If it is made for each stone separately, generally the phosphate is adequately accounted for as  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{Mg NH}_4 \text{PO}_4$ , but in some stones there is an excess of  $\text{P}_2\text{O}_5$  and in others a deficiency. This is probably due to the existence of other phosphates of calcium in the stones besides  $\text{Ca}_3(\text{PO}_4)_2$ , and of other calcium compounds. In some of the stones a small percentage of carbonate was present this is presumably in the form of calcium carbonate. In most of the stones there is some sulphate which may well be in the form of calcium sulphate.

Reasons were given in the previous report for considering that on the average about 1 per cent of the nitrogen is in the form of protein. A little is in the form of ammonium and to correspond to 2.0 per cent of  $\text{MgO}$  in the form of magnesium ammonium phosphate 0.35 per cent would be needed. Subtracting  $1+0.35$  per cent from the total nitrogen in the average stone (17.9 per cent) leaves 16.55 per cent of nitrogen as uric acid.

The approximate composition of the average stone is therefore —

Calcium oxalate	24.9
Calcium phosphate	7.4
Magnesium ammonium phosphate	6.9
Protein	6.2
Uric acid	49.7
	<hr/>
	95.1
Difference from 100	4.9
	<hr/>

The unaccounted for residue is due to carbonates and insoluble ash in a few stones, a trace of sulphates in most of them, water which is not driven off at  $100^\circ\text{C}$ , sodium, potassium, hydrogen in combination with nitrogen, and other undetermined substances.

#### CLASSIFICATION

In the previous report following the usual practice the stones were classified into urate, phosphate and oxalate stones and mixtures of these. Amounts of phosphates and oxalates below 1 per cent were neglected and the murexide test was used for deciding if stones with only a little nitrogen were to be classed as containing some urate or not. The results of classifying all the vesical stones by this method are shown in Table V.

TABLE V  
*Classification as for first 100*

	U	P	O	UP	UO	PO	UPO	TOTAL
N W F P	4	1	2	4	11	2	7	31
Punjab	4	0	1	5	9	0	5	24
United Provinces	2	0	0	4	23	6	10	44
Central Provinces			1		2	2	2	7
Bengal			1	1		2	2	6
Assam	2	0	2	3	22	3	33	65
Ajmeer Meeerwar				1	1			2
Madras		1	1			4		6
Bombay	1	0	0	1	8	4	11	25
Behar and Orissa	1				1		1	3
Nepal							2	2
Hyderabad	1	1		1	1	1	1	6
	15	3	8	20	76	23	74	221
Per cent	6.8	1.4	3.6	9.1	36.3	10.4	33.4	

Urate in	187	84.6	per cent
Phosphate in	120	54.3	"
Oxalate in	183	82.8	"

It will be noticed that only 26 stones out of 221 (11.8 per cent) consist purely of one of these substances. The remainder consist of mixtures, of which a mixture of urates and oxalates is the most common. In 187 of the stones (84.6 per cent) some urate was present, in 120 (54.3 per cent) some phosphate and 183 (82.8 per cent) some oxalate. A mixed stone is the usual thing.

Another method of classifying stones is to group them according to their main constituent. It has been shown in the previous report that on the average 1 per cent of the nitrogen is in the form of protein and the rest mostly uric acid. For a stone to contain 50 per cent or more of uric acid, there must be  $\frac{1}{3} \times 1 = 17.7$  per cent or more per cent of nitrogen. (Since uric acid contains one-third of its weight of nitrogen.)

Similarly 50 per cent of calcium phosphate requires 22.9 per cent  $P_2O_5$  and more if magnesium phosphate is present, and 50 per cent of calcium oxalate requires 28.1 per cent of  $C_2O_3$ .

Classing the stones in this way leaves but a small class of 40 stones in which no constituent exceeds 50 per cent of the stone. This classification is shown in Table VI.

TABLE VI

*Classification by over 50 per cent of one constituent    Numbers of stones*

	Uric acid	Phosphates	Oxalates	Mixed	TOTAL
N W F P	15	3	6	7	31
Punjab	13	6	3	2	24
United Provinces	30	5	5	4	44
Central Provinces	3	0	1	3	7
Bengal	0	1	2	3	6
Assam	19	5	12	9	65
Ajmeer Meerwar	2	0	0	0	2
Madras	0	2	1	3	6
Bombay	11	1	7	6	25
Behar and Orissa	2	0	0	1	3
Nepal	0	0	0	2	2
Hyderabad	3	1	0	0	6
All	118	26	37	40	221

*As percentages of all the stones from each province*

N W F P	48	10	10	23
Punjab	54	25	13	8
United Provinces	68	11	11	9
Central Provinces	43		14	43
Bengal		17	33	50
Assam	60	8	18	14
Ajmeer Meerwar	100			
Madras		33	17	50
Bombay	44	4	28	24
Behar and Orissa	67			33
Nepal				100
Hyderabad	50	50		
All stones	53	12	17	18

## THE KIDNEY STONES

Out of the 226 stones received in this laboratory in connection with this Inquiry only 5 were kidney stones. The proportion must not be taken as an accurate measure of the actual frequency of the two kinds of stones in India since, as this was primarily an investigation into stone-in-the-bladder, kidney stones may not have been sent. It does however reflect the common observation that, in India, stone is very much more prevalent in the bladder than in the kidney. In Europe and America the opposite seems to be true, viz., that kidney stones are much more common than bladder stones. According to Swift Joly(3) vesical calculus, though formerly common in England, is now comparatively rare, while the incidence of renal calculus remains about the same. In searching such of the literature of stone as is available here, 71 quantitative analyses of kidney stones have been found and only 5 of bladder stones.

The five kidney stones form a class apart not only in their origin but in their composition. They are all essentially calcium oxalate stones. In only one (94) was any urate present and in that but little. Their composition is given in Table VII. So far as can be judged from 5 specimens the composition of Indian kidney stones is not different from kidney stones in other countries. They are however definitely different from bladder stones, and statistically, the odds, so far as they can be estimated from these 5 specimens, are more than 100 to 1 that kidney and bladder stones in India are essentially different in composition. On the other hand Indian bladder stones are not demonstrably different in composition from the 5 bladder stones from other countries.

TABLE VII

*Composition of the kidney stones*

Stone number	N	P <sub>2</sub> O <sub>5</sub>	CaO	MgO	C <sub>2</sub> O <sub>3</sub>	Age
110	0.6	0.8	33.8	0	45.9	43
151	0.9	0.7	34.9	0	37.0	12
53	0.9	4.7	36.6	0	35.3	29
154	1.4	12.8	37.7	1.6	35.5	25
94	5.5	0.7	30.7	0	44.8	30
Means	1.9	3.9	34.5	0.3	39.7	28

## THE DIAGNOSIS OF THE COMPOSITION OF A STONE BY INSPECTION

In stones consisting of only one substance a very good guess as to their composition can generally be made by inspection. If on the other hand the stone

consists of a mixture of substances, as is usually the case, it is much more difficult even when the stone is cut across and found to be layered. An experiment was tried to see with what accuracy a diagnosis could be made by inspection. A series of typical stones was put up and their exact composition given. With this series as a reference various people were asked to diagnose the composition of another series of 30 stones selected haphazard from amongst our collection. The classification required was into uric acid or urates, phosphates and oxalates as the chief constituent. The numbers diagnosed correctly varied from 12 to 22, and it must be remembered that if the diagnosis were made purely haphazard without any reference to their appearance one would expect on the average 10 right out of 30. The surgeons who sent the stones to us often offered an opinion as to their composition and were scarcely more successful than were the persons tried in our test. It is difficult to decide what is to be considered a correct diagnosis. It is obviously unreasonable to expect any one to distinguish 1 per cent of any constituent or even 5 per cent. The most reasonable figure to take would seem to be about 20 per cent, that is to say that in classifying the stones amounts of any constituent less than 20 per cent should be neglected. On this basis out of 99 stones in which the surgeon offered an opinion as to their composition he was right in 26, partly right in 24 and wrong in 49. In 59 of the stones the diagnosis offered was a single constituent and in these stones it may be fairer to assume that here an attempt was only made to decide the main constituent, i.e., the constituent forming more than 50 per cent of the stone. If this is the meaning, the diagnosis was right in 30 and wrong in 29. If one takes a diagnosis of one constituent to mean the main constituent and judges the diagnosis of two constituents to be right or wrong by neglecting constituents present to the extent of less than 20 per cent then in the whole 99 stones the surgeon was correct in 36, partly correct in 4 and wrong in 59. It is quite safe to conclude that the diagnosis of the composition of a stone from its appearance is of very little value.

#### SUMMARY

1 In this paper and in the previous report(1) taken together the analyses of 221 vesical calculi and 5 renal calculi collected from all parts of India are given

2 The approximate composition of the average vesical calculus is —

Calcium oxalate	24.9
Calcium phosphate	7.4
Magnesium phosphate	6.9
Protein	6.2
Uric acid	49.7
Other substances	4.9
	<hr/> 100.0 <hr/>



3 The vesical calculi showed great variations in composition, and the great majority consisted of a mixture of substances the most common mixture being a mixture of urates or uric acid and oxalates (35.3 per cent). In 84.6 per cent of the stones some urate or uric acid was present. Of the pure stones uric acid stones were the most common (6.8 per cent of all stones).

4 If the main constituent of the stones is alone taken into consideration, then urate stones are the most common (53 per cent), oxalate stones next (17 per cent) and phosphate stones least common (12 per cent). The remainder (18 per cent) being stones with no main constituent.

5 The five kidney stones were all oxalate stones.

6 The determination of the composition of a stone from its appearance is generally very inaccurate.

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# THE COMPOSITION OF URINARY CALCULI IN RATS

BY

MAJOR CLIVE NEWCOMB, D M , I I C , I M S ,

AND

S RANGANATHAN, B A , A I I S c ,

*From the Nutritional Research Laboratories I R F A , Pasteur Institute, Coonoor  
S India*

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A SERIES of twenty-two urinary calculi, occurring in white rats, kept on various deficient diets, have been analysed and the results are reported in this paper. Twenty-one of these stones were vesical and one was a renal stone.

The stones were all very small in absolute weight [mean weight (wet) 20.7 mg., range 3.4 to 66.6 mg.] but comparatively to the size of the animal, many would correspond to large human stones. The smallness of the stones necessitated special micro-methods of analysis and the analytical scheme adopted was that described in a previous paper (Newcomb, 1930). It was carried out so far as the amount of material allowed. The results are shown in Table I. The diets which the rats were receiving are given in Table II.

TABLE I

*Showing the results of the analyses of rats' stones, in percentages of dry stone  
(except moisture)*

Stone No.	Diet	Moisture	Total N	P O <sub>2</sub>	CaO	MgO	C O <sub>2</sub>	Murexide test	Carbonate
			15	19	5	7	—	0	+ +
			5	41	—	13	—	0	+ +
				27	1	12	—	0	? +
				25	1	7		0	? +

( 1055 )

TABLE I—*concl'd*

Stone number	Diet	Moisture	Total N	P <sub>2</sub> O <sub>5</sub>	CaO	MgO	C <sub>2</sub> O <sub>3</sub>	Murexide test	Carbonate
Bladder	Stones								
5	F	34	8	23	1	11		0	+
6	G	38	12	34	1	12		0	+ +
7	A	41	15	32	+	10	1	0	+ +
8	B	(9)	16	16	1	9		0	+ +
9	C	43	5	23	+	8		0	+ +
10	D	43	9	48	4	25	1	0	+ +
11	F	42	7	28	+	15	4	0	+ +
12	I	38	10	46	8	22		0	+ +
13	A	43	7	34	+	14		0	+ +
14	B	40	7	49	+	30		0	+ +
15	D	38	5	37	+	22		0	+ +
16	E	44	5	46	+	20		0	+ +
17	J	40	5	32	+	11	+		+ +
18	K	37	10	19	+	5	+	0	+ +
19	J	42	5	36	+	17	+	0	+ +
20	L	40	6	40	+	10	+	0	+ +
21	K	41	6	41	+	10	+	0	+ +
Means	-	38	8	33	1	14	+	0	+ +
Kidney 22	Stone M		5	1	38	2	9	0	+ --

The figures for the moisture total nitrogen and phosphate are thought to be accurate to within 1 per cent or so. The figures for the calcium, magnesium and oxalates must be taken more as indications of the approximate amounts present than as accurate determinations, especially the magnesium. The figures for the last are probably all low.

TABLE II

*Showing the composition of the various diets used*

	Per cent		Per cent
A	Whole wheat flour	I	Whole wheat flour
	Corn flour		53
	Linseed meal		24
	Calcium phosphate		20
	Sodium chloride		1
B	Oatmeal	J	Oatmeal
	Corn flour		53
	Linseed meal		20
	Calcium phosphate		White flour (later changed to corn flour)
	Sodium chloride		25
C	White flour	K	Calcium phosphate
	Corn flour		1
	Linseed meal		Sodium chloride
	Calcium phosphate		1
	Sodium chloride		1
D	Whole wheat flour	L	White bread
	Linseed meal		97
	Calcium phosphate		Dried yeast
	Sodium chloride		3
E	Whole wheat flour	M	White bread
	Linseed oil		97
	Calcium phosphate		Dried yeast
	Sodium chloride		3
F	Whole wheat flour	N	+ 5 grains of slaked lime + 5 drops of a solution containing 1 milligram of iodine in 1 litre of distilled water
	Gingelly oil		Cascia
	Calcium phosphate		80
	Sodium chloride		Olive oil
G	White flour		8
	Olive oil		Salt mixture
	Salt mixture		5
			Cod liver oil
			2
			Yeast
			2

We are led to this conclusion from a consideration of two analyses of the sediment which is deposited in rats' urine on standing. This can be collected in comparatively large quantities and is very similar to rat stones in its composition.

The results of these two analyses by macro-methods are shown in Table III. The first was from the mixed urines of rats on various diets and the second from rats on a high protein diet (Diet 'N' in Table II)

TABLE III

*Showing the results of analyses of two sediments from rats' urine*

	I	II
Ash	70.7	55.4
Volatile matter by difference	29.3	44.6
Nitrogen	4.2	5.6
Insoluble ash	0.38	0
CaO	3.48	9.3
MgO	23.20	12.96
K <sub>2</sub> O	2.38	3.26
P <sub>2</sub> O <sub>5</sub>	41.80	31.0
SO <sub>4</sub>	.0	0
Total ash accounted for	71.24	56.52
CO <sub>2</sub>	3.25	3.12

The analytical figures for both these analyses are consistent with the following assumptions —

- 1 The oxalate is present as calcium oxalate
- 2 The remaining calcium is present as calcium phosphate  $[\text{Ca}_3(\text{PO}_4)_2]$
- 3 The magnesium is present as magnesium ammonium phosphate

When the micro-method was tried on a few milligrams of this sediment the nitrogen and phosphate came out in agreement with the large scale determinations but the other figures were only approximately in agreement, especially the magnesium, which always came out too low.

The following points about the rat stones are notable —

- 1 The bladder stones are all essentially magnesium ammonium phosphate stones with small quantities of other substances of which carbonate is the chief
- 2 The bladder stones contain very little calcium
- 3 None of the stones contain uric acid or urates
- 4 The one kidney stone is of very different composition to the bladder stones being essentially a mixture of calcium carbonate with a little oxalate

- 5 The kidney stone occurred on the only diet to which slaked lime had been added

The numbers of stones analysed on the different diets is insufficient for determining any but very gross connections between diet and composition. One association is to be noticed though its statistical significance is not very definite ( $P=7$  per cent) viz, that the stones occurring on a diet containing wheat as opposed to a diet containing oatmeal have more nitrogen.

Comparing these rat bladder stones with Indian human bladder stones (Newcomb 1929) the chief differences to be noticed are --

- 1 In human stones the most common constituent is uric acid or urates and in these rat stones this is entirely absent
- 2 On the average human stones contain 14.9 per cent of CaO and 2.0 per cent of MgO while rat stones contain 1.1 per cent of CaO and 13.8 per cent of MgO
- 3 Human stones contain much oxalate and little carbonate and rat stones much carbonate and little oxalate
- 4 Rat stones contain much more  $P_2O_5$  than human stones

A comparison of the mean composition of human and rat vesical stones is given in Table IV

TABLE IV

*Giving a comparison between the average composition of rat and human vesical stones in India*

	Moisture	N	$P_2O_5$	CaO	MgO	$CO_2$
Rat stones	37.8	8.3	33.1	1.1	13.8	0.3
Human "	7.2	17.9	6.9	11.9	2.0	14.0

## REFERENCES

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# LEVEL OF IODINE-METABOLISM, INSANITARY CONDITIONS OF LIFE AND GOITRE

BY

COLONEL R McCARRISON, C I E , K H P M D , F R C P , I M S ,  
*Director, Nutritional Research, I R F A Pastur Institute, Coonoor, S India*

WITH

A STATISTICAL EXAMINATION OF THE EXPERIMENTAL DATA

BY

MAJOR CLIVE NEWCOMB, D M F I C , I M S

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## Introduction

THE original purpose of the investigation with which this paper deals was to determine the effect on the thyroid gland of a diet having a low content of iodine. But as the work progressed it took on a wider aspect, hence the title of this paper.

Within recent years a number of attempts have been made to determine the effect on the thyroid gland of diets having a low iodine-content.

McClendon and Williams(1) (1923) performed experiments on rats in which they state that the iodine-content of the dry food was less than 120 parts per billion. More accurate analyses later showed it to contain 10 parts per billion of iodine. The rats receiving this low-iodine diet and distilled water developed goitre, whereas control rats receiving the same diet and water containing 0.01 per cent of iodine had normal thyroids. The size of the thyroids in the former group was approximately twice that of the latter.

More careful experiments were made by Hayden, Wenner and Rucker (1924) (1)(2). These observers used a diet consisting of 53 parts of oatmeal, 25 parts of patent flour, 20 parts of linseed meal, one part of calcium phosphate, one part of sodium chloride, and distilled water *ad libitum*. Rats fed on this diet developed goitre 'within a few weeks,' while control rats fed on the same diet but receiving



distilled water containing 0.1 mg of iodine per litre, remained free from thyroid enlargement. The size of the thyroid in those which received no additional iodine was approximately twice that of those which received iodine. The difference in size of the gland in the two groups was determined by weight. Twelve rats were used in the experiment, and the glands were removed at different intervals up to 60 days during the course of the experiment. No histological study of the thyroids appears to have been made, or if made, and reported the records are not available in this Institute. This 'oatmeal diet' was stated to contain only 9γ of iodine per kilogram of the dry food.

Heicus and Roberts(3) (1927) 'have also found that the thyroids of animals fed on deficient iodine show enlargements up to three times the normal size. Histologically, such glands were with one exception not found to be definitely pathological being in the main of the simple colloid type of enlargement'—an interesting observation since compensatory hyperplasia of the thyroid is assumed to be 'immediately dependent upon a relative or absolute deficiency of iodine'(4) 'In one or two cases a marked degree of hyperplasia was also shown the glands thus being of the actively secreting type'(3) 'The thyroids of white rats fed for some months on a diet of white bread and a little milk were also twice the normal size, and in one case consisted partly of a congested hyperplastic area, and partly of a group of very large acini filled with dense colloid mixed with cells'(3) 'In all cases the iodine-content of the gland, in milligrams per gramme, was abnormally low'(3) The iodine-content of the foods used is not stated in the paper(3) from which these details are taken.

In all these experiments the diets used were faulty in other regards besides their low content of iodine. The results do not, therefore, provide evidence of the effects on the thyroid gland of partial deficiency of iodine *per se*. Nor do they afford definite evidence that the goitres arising during the course of the experiments were due to the direct action on the thyroid gland of iodine-deficiency. They do, however, appear to show that whatever may have been the cause of the goitre, a low intake of iodine favoured, while the additional provision of iodine counteracted its action.

In all attempts to ascertain the effects on the thyroid gland of iodine-deficiency *per se*, there is always this problem before the investigator: the devising of a diet which while sufficiently low in iodine will not also be lacking in other elements and complexes necessary for normal nutrition. Up to the present no such diet has been devised, and until it has, and until its action has been tested under carefully controlled conditions on a sufficiently large scale, the effects of iodine-deficiency *per se* on the thyroid gland must remain unknown. It is not uncommon to come across, in current medical literature, statements to the effect that 'goitre is due to absolute or partial deficiency of iodine' or that 'deprived of an element necessary for its normal functioning the thyroid gland undergoes compensatory hypertrophy'(5). Such statements are not strictly in accord with the known facts. There is no such thing as absolute deficiency of iodine in the diets

customarily consumed by mankind while as indicated above the effects of iodine deficiency *per se* on the thyroid gland are as yet unknown, as also are its effects on the body tissues generally. Deficiency of iodine relative to certain goitrogenous agencies is however a different matter, and it is here that an abnormally low intake of iodine may be of importance in regard to the genesis of 'goitre'.

The experiment of Hayden, Wenner and Rucker was repeated in this laboratory on a scale sufficiently large to make statistical analysis of the results possible.

### Composition of the experimental diet

The ingredients of the diet and the proportions in which they were combined, were the same as in the experimental diet used by Hayden, Wenner and Rucker, but the sources from which the ingredients were obtained were, of necessity, different. A difference in the iodine content of the diet, as used by these observers and as prepared in this laboratory was therefore to be expected, though it was not expected to be so great as it proved to be. The composition of the diet is shown in Table I together with the source from which each ingredient was derived. Its chemical composition is given in Table II. The diet is markedly deficient in vitamin A.

TABLE I

*Showing the composition of the experimental diet and the source of each ingredient*

Ingredient	Parts per 100	Source
Coarse Scotch oatmeal	53	Scaled tins. Crosse and Blackwell London
Patent Corn flour	25	Scaled tins. Brown and Polson, Paisley, England
Linseed meal	20	Freshly ground seeds. Madras
Sodium Chloride (pure)	1	Riedel, Berlin
Calcium Phosphate (pure)	1	Mayer and Bailer, London
Distilled water	<i>ad lib</i>	Freshly distilled daily from local water supply
Iodized distilled water	<i>ad lib</i>	

TABLE II

*Showing the chemical composition of the experimental diet .*

Constituents	PERCENTAGE	
	Expressed on original sample	Expressed on moisture free sample
Moisture	20 50	
Mineral Matter	5 73	7 20
Ether Extractives	10 45	13 14
Crude Protein	9 705	12 26
Crude Fibre	1 06	1 32
Carbohydrates	52 555	66 08
TOTAL	100 000	100 00
Albuminoids	9 36	11 77
Insoluble Mineral Matter	0 37	0 465
Soluble Mineral Matter	5 36	6 735
TOTAL	5 73	7 2
Soluble Silica	0 0363	0 0456
Phosphoric Acid ( $P_2O_5$ )	1 451	1 82
Iron Oxide ( $Fe_2O_3$ )	0 046	0 058
Alumina ( $Al_2O_3$ )	0 366	0 462
Manganese Manganic Oxide ( $Mn_2O_3$ )	0 0014	0 00176
Lime (CaO)	1 08	1 38
Magnesia (MgO)	1 05	1 32
Potassium Oxide ( $K_2O$ )	0 21	0 264
Soda ( $Na_2O$ )	0 904	1 13
Sulphuric Acid ( $SO_3$ )	0 19	0 23
Undetermined	0 025	0 024
TOTAL	5 36	6 735

The iodine-content of this diet was estimated by the method of v Fellenberg on three occasions. On first analysis it was found to be 20γ per kilogram of the dry food. Subsequent analyses yielded higher figures—125 and 150γ—and these are regarded as being nearer the truth though probably on the high side as judged by the urinary excretion of iodine by rats fed on the diet (*vide infra*). It contained, however considerably more iodine than 9γ per kilogram, the amount said to be present in the diet used by Hayden, Wenner and Rucker. The variation in iodine-content of a food-material, such as oatmeal, is known to be considerable and to depend to some extent, on its place of origin. Thus in Minnesota(1) oats are said to contain only 10γ of iodine per kilogram, in Storrs, Conn 23γ(1), in Wisconsin Me 175γ(1) in Fayetteville, Ark, 3,110γ(6), in Fargo, N Dak, 3,330γ(6) and in Wibaux Mont 5,800γ per kilogram of the dry material(6). Fresh oatmeal in Germany(7) is reported to contain 11γ of iodine per kilogram and in New Zealand 30γ(3). It may be therefore, that the Scotch oatmeal used in this laboratory was considerably richer in iodine than that used by Hayden, Wenner and Rucker and that the other ingredients of the diet varied in the same way.

A considerable experience of iodine estimations in foodstuffs, by the method of v Fellenberg(9) has caused us to have grave doubts as to their accuracy when the amount of iodine present is less than 100γ per kilogram of the dry material. The estimation of iodine in urine is not fraught with the same technical difficulties and fallacies and we consider that 20γ per litre can be estimated with comparative accuracy. Accordingly, we have abandoned the estimation of iodine in the experimental diets used for thyroid work in this laboratory, and have adopted the concentration of iodine in the urine as an index of the level of iodine-metabolism. This index furnishes a reliable numerical standard whereby to gauge the effect on the thyroid gland of different levels of iodine-metabolism, and a means of comparing the results observed in laboratory experiments with those obtained by estimation of the concentration of iodine in the urine of goitrous and non-goitrous human beings.

### Urinary excretion of iodine by rats fed on the experimental diet

For the purposes of this estimation four previously well-fed rats, of an average body-weight of 168 grammes, were used. They were taken from stock, transferred to metabolism cages, and fed on the experimental diet and distilled water. After the lapse of several days, to permit of iodine-equilibrium being established on the new diet, the concentration of iodine in the urine was determined each day for seven consecutive days. The results are given in Table III.

The average urinary excretion of iodine per rat per day was thus found to be 0.033γ per cc, or 33γ per litre, in those receiving the experimental diet *without iodized water*.

TABLE III

*Showing the urinary excretion of iodine by 4 rats fed on the experimental diet*

Day	Volume of urine c c	$\gamma$ of iodine in total urine	$\gamma$ of iodine per c c urine
1st	32.3	1.75	0.055
2nd	42.0	1.5	0.036
3rd	46.4	1.75	0.038
4th	55.4	1.75	0.032
5th	50.9	0.8	0.016
6th	60.2	1.3	0.022
7th	39.4	1.2	0.031
Average per rat per day	11.4	0.36	0.033

The iodized water used in the first experiment contained 0.1 mg, or 100 $\gamma$ , of iodine per 1,000 c c. Of this the animals drank on the average 10 c c daily or an ingestion of 1 $\gamma$  of iodine additional to that present in the experimental diet. It has been found, in this laboratory, that of the iodine administered as such approximately 60 per cent is recoverable from the urine, so that of the 1 $\gamma$  of iodine ingested as iodized water, 0.6 $\gamma$  would appear in the urine. With an average excretion of 11.4 c c of urine daily this would amount to 0.052 $\gamma$  per c c, or 52 $\gamma$  per litre. The latter figure, added to the 33 $\gamma$  per litre excreted by rats not receiving iodized water, makes the average urinary excretion of iodine by rats fed on the experimental diet with iodized water equal to 85 $\gamma$  per litre.

The iodized water used in the second experiment contained 10 times as much iodine as that used in the first. The urinary excretion of iodine by the rats which received the experimental diet with this iodized water is estimated to have averaged 559 per litre.

The urinary excretion of iodine by well-fed stock rats in this laboratory, amongst which goitre is unknown, is from 56 to 85 $\gamma$  per litre.

It is of importance in connexion with the experimental results, presently to be recorded, to compare these figures with those recorded for goitrous and non-goitrous persons in various parts of the world.

*Urinary excretion of iodine in  $\gamma$  per litre*

	NON GOITROUS PERSONS		GOITROUS PERSONS	
	Range	Average	Range	Average
* Switzerland(21)	36—72	42.6	2.6—19.3	12
* Italy(21)	20—93	48		
* Norway(21)		115	4—93	35.8
* Oslo(22)		115		26.2
New Zealand(3)	11—384	49	5—75	25

From these figures it is seen that the urinary excretion of iodine (33 $\gamma$  per litre) by rats fed on the experimental diet *without iodized water* falls within the range recorded, in these localities for goitrous persons, while that (85 $\gamma$  per litre) of rats fed on the same diet *with iodized water* is well above the average for goitrous persons. So far, then, as its iodine content or more properly, its capacity to sustain iodine-metabolism, is concerned the experimental diet with and without the addition of iodized water, simulated closely the diets in use by non-goitrous and by goitrous persons in certain parts of the world. We are, therefore, in a position to learn, from the following experiments the effects on the thyroid gland of levels of iodine-metabolism similar to those of goitrous and non-goitrous persons. But since the work of McCarrison(8)(10), Marine(11), Lenhart(11), Gaylord(12), Sasaki(13), Burget(14), and others has established a connexion between unsanitary conditions of life, and thyroid enlargement, it was necessary to examine the effects on the gland of these levels of iodine-metabolism under two distinct conditions of life (a) hygienic and (b) unhygienic.

### The experiments

Two experiments, involving the use of 180 animals, were carried out the first in the summer months of 1926, the second in the summer months of 1929.

The animals used were white rats from non-goitrous stock whose diet was not poor in iodine. The possibility of inherited goitrous taint, or of increased susceptibility to thyroid disease consequent on the insufficient provision of iodine in the food prior to the commencement of the experiments, was thus eliminated a very important precaution in experiments of this kind. It is to be noted that the albino rats used in this laboratory are of a smaller variety than those used in nutritional laboratories in the United States of America and in Great Britain.

\* Figures calculated from those given for daily excretion of iodine on the assumption that the average daily excretion of urine is 1.5 litres.

The animals selected were aged between 40 to 60 days at the outset of the experiments, those in the first experiment being the younger. The sexes were equally distributed in the various experimental groups. In both experiments the animals were caged in colonies.

*Hygiene* Some of the animals were confined in 'clean cages' under conditions of meticulous cleanliness, others in 'dirty cages' having wooden floors (instead of screens) on which the excreta were allowed to accumulate and to contaminate the food and drinking water. The cages of the animals living under clean conditions were frequently sterilized by submersion in boiling water and by cleansing, in the intervening periods with antiseptic lotion. Attendants removed, by hand, any faecal matter which adhered to the netted-wire screens forming the bottom of the cages. Without minute attention to these details the influence of 'dirt' in causing goitre cannot be excluded. The rats were given no bedding since their tendency to eat the cotton-waste usually provided as bedding, would have introduced another factor which might have complicated the results.

Half the animals in each experiment were given distilled water and the other half iodized distilled water. The former were kept in one animal house the latter in another at a distance from the first. Both groups had their own attendants, feeding vessels, etc. In these ways possible sources of ingress of iodine, into the food of animals not intended to receive it, were eliminated.

#### **First experiment . commenced 16-6-1926.**

In this experiment 120 young rats were used, 64 females and 56 males. Their ages at the outset of the experiment were between 40 and 45 days, and their weights between 45 and 60 grammes. They were divided into two main groups of 60, the one received the experimental diet and distilled water, the other the experimental diet and distilled water containing 0.1 mg., or 100 $\gamma$ , of iodine per litre. Each group was housed in four roomy cages, 15 animals, 8 females and 7 males, in each cage. The cages used were identical as to size (36"  $\times$  18"  $\times$  18"). Thirty animals in each group were confined in screened cages and lived under the 'clean' conditions previously described, 30 in each group were confined in cages having wooden floors and lived under the 'dirty' conditions previously described. There were thus four sub-groups as follows—

1	Clean cages with iodine	30 animals, 16 females and 14 males
2	Dirty cages with iodine	" " " "
3	Clean cages without iodine	" " " "
4	Dirty cages without iodine	" " " "

On the 35th day, the 49th day and the 64th day of the experiment four animals from each of these sub-groups were killed, two males and two females, care being taken to select animals of as nearly the same weight as possible—a desideratum which became increasingly difficult as the experiment advanced. The larynx and

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\* Added to the water in the form of Lugol's solution

trachea with the attached thyroid were removed from each animal. Eight of the specimens—two from each sub-group—were transferred to fixative for subsequent serial sectioning. The thyroids of the remaining eight were dissected out, under a magnifying lens and weighed, precautions being taken to prevent drying. The weights of the thyroids removed on the 35th, the 49th and the 64th days are given in Tables IV, V and VI.

TABLE IV

*Showing weights of thyroids of 8 animals on the 35th day of experiment*

Cages	Iodine	Sex	Body weight in grammes	Weights of thyroids in mg	Average weight in mg per 100 grammes of body weight
Clean	No	M	110	9	8.2
Clean	No	F	80	5	6.3
Dirty	No	M	110	9	8.2
Dirty	No	F	80	7	8.8
Clean	Yes	M	110	6	5.5
Clean	Yes	F	85	6	7.1
Dirty	Yes	M	110	7	6.4
Dirty	Yes	F	80	6	7.5

TABLE V

*Showing weights of thyroids of 8 animals on the 19th day of experiment*

Cages	Iodine	Sex	Body weight in grammes	Weights of thyroids in mg	Average weight in mg per 100 grammes of body weight
Clean	No	M	113	10.8	9.6
Clean	No	F	100	8.0	8.0
Dirty	No	M	113	9.0	8.8
Dirty	No	F	100	9.8	9.8
Clean	Yes	M	115	7.0	6.1
Clean	Yes	F	100	8.6	8.6
Dirty	Yes	M	115	11.0	9.6
Dirty	Yes	F	100	8.2	8.2



TABLE VI

*Showing weights of thyroids of 8 animals on the 64th day of experiment*

Cages	Iodine	Sex	Body weight in grammes	Weights of thyroids in mg	Average weight in mg per 100 grammes of body weight
Clean	No	M	100	90	90
Clean	No	F	90	80	89
Dirty	No	M	100	90	90
Dirty	No	F	90	100	111
Clean	Yes	M	100	80	80
Clean	Yes	F	90	70	78
Dirty	Yes	M	100	80	80
Dirty	Yes	F	95	70	74

Taking all three Tables (IV, V and VI) together, the mean weights of the thyroids, expressed as mg per 100 grammes of body-weight for each of the four sub-groups are shown in Fig 1. From this Figure it will be seen that the thyroids of animals living in clean cages and receiving iodized distilled water were the smallest, those living in dirty cages and receiving distilled water without added iodine were the largest, the other two sub-groups occupying an intermediate position between these two extremes.

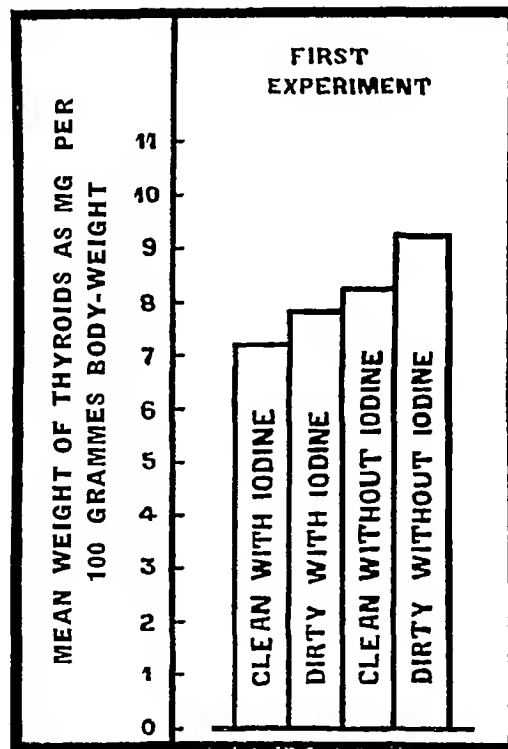


FIG 1.

After the last killing on the 64th day, the experiment was continued until the death from required disease, of all remaining animals. The last animal died on the 157th day.

### Second experiment commenced 8-5-1929

The details of the experiment were the same as those of the first, except in the following regards. 61 animals were used and these were slightly older—50 to 60 days—and their initial body-weights were proportionately greater than those in the first experiment, the iodized water used contained 1 mg of iodine per litre, added in the form of potassium iodide, and, the experiment was allowed to proceed to the 60th day before the animals were killed.

A noticeable feature of this experiment was the higher mortality amongst the animals not receiving iodized distilled water. The duration of the experiment was limited by this mortality, for by the 60th day there remained alive only 8 animals, out of the original 16, in each of the two sub-groups receiving no iodine, while there were 27 survivors out of the original 32, in the two sub-groups receiving iodine. 14 in the dirty and 13 in the clean cages. The chances are more than 100 to 1 that this result did not arise by simple sampling.

Eight animals from each sub-group were killed on the 60th day of the experiment and their thyroids dissected out and weighed. The remaining animals were spared and the experiment allowed to proceed until their death from acquired disease. The weights of the 32 thyroids thus made available for comparison are shown in Table VII. One animal (dirty iodine), which died on the 54th day, is included with the 8 members of its sub-group killed on the 60th day.

TABLE VII

*Showing weights of thyroids of 32 animals on the 60th day of experiment*

Conditions		Body weight in grammes	Thyroid weight in mg	Weight of thyroid in mg per 100 grammes of body weight
1	Clean cages without iodine	54	5.2	9.6
2	" "	92	9.0	9.8
3	" "	106	8.0	7.5
4	" "	93	7.0	7.5
5	" "	70	6.4	9.1
6	" "	55	4.8	8.7
7	" "	45	5.0	11.1
8	" "	72	8.4	11.6

TABLE VII--*concl'd*

Conditions	Body-weight in grammes	Thyroid weight in mg	Weight of thyroid in mg per 100 grammes of body-weight
1 Dirty cages without iodine	58	5.4	9.3
2 " "	78	8.6	11.0
3 " "	87	9.2	10.5
4 " "	87	10.8	12.4
5 " "	64	7.2	11.2
6 " "	87	9.8	11.3
7 " "	88	9.6	10.9
8 " "	93	10.2	11.0
1 Clean cages with iodine	83	7.8	9.4
2 " "	100	9.0	9.0
3 " "	78	9.4	12.0
4 " "	65	8.4	12.9
5 " "	90	9.2	10.2
6 " "	70	6.6	9.4
7 " "	73	7.0	9.6
8 " "	63	7.8	12.4
1 Dirty cages with iodine	76	8.8	11.6
2 " "	83	8.8	10.6
3 " "	139	10.8	7.8
4 " "	106	8.6	8.1
5 " "	80	6.8	8.5
6 " "	79	9.2	11.6
7 " "	68	8.0	11.8
8 " "	86	7.0	8.1
*9 " "	66	5.2	7.9

\* Died on the 54th day, but included with others killed on 60th day

The mean weights of the thyroids of the animals in the four sub-groups, calculated in mg per 100 grammes of body-weight, are shown in Fig 2. It will be seen from this Figure that the thyroids of animals living in clean cages and receiving no additional iodine were the smallest, those of animals living in dirty cages without additional iodine the largest—the two remaining groups occupying an intermediate position between these extremes.

The combined results of the two experiments are shown in Fig 3.

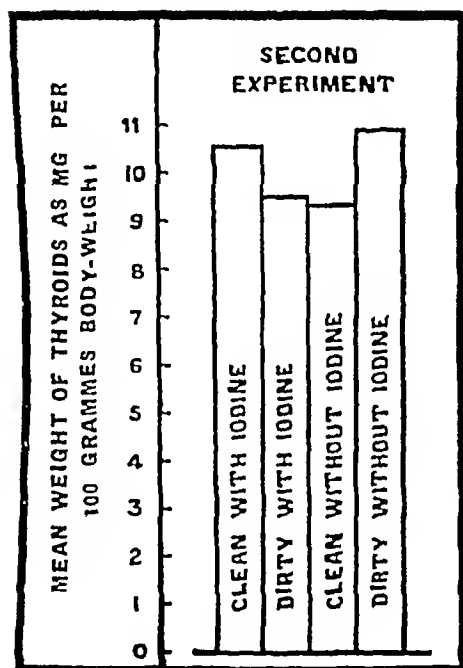


FIG 2

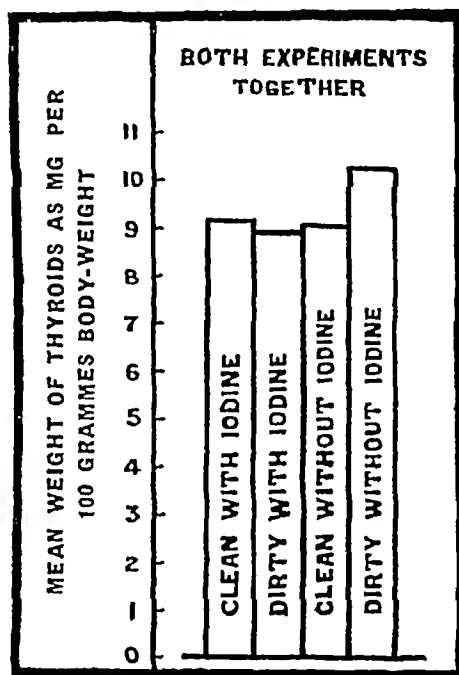


FIG 3

### Results of the experiments

In an Appendix to this paper the statistical significance of the results of these two experiments is discussed independently by Major C Newcomb.

In both experiments the thyroids of the animals living under insanitary conditions ('dirty cages') were appreciably larger than those of animals living under sanitary conditions of life ('clean cages'), neither group having received additional iodine. It would seem, therefore, that the insanitary conditions were the cause of the thyroid enlargement. The operation of this goitrogenous agency was not counteracted by a level of iodine-metabolism represented by 33γ of iodine per litre of urine. In the first experiment the administration of iodized water, and the raising thereby of the level of iodine-metabolism from 33γ to 85γ of iodine per litre of urine, appeared to counteract the goitrogenous influence of 'dirt' but the difference in size of the glands in the two groups was not great and if this

experiment were to stand alone one would remain in doubt both as to the goitrogenous effect of 'dirt' and as to the anti-goitrogenous effect of the iodized water. But in the second experiment the result is more definite and is confirmatory of the first. It is confirmatory also of the previous work of McCarrison (8), (10), Maine(11), Gaylord(12), Burget(14) and others, whereby a connexion between insanitary conditions of life and thyroid enlargement was established. It may, therefore, be concluded that the additional provision of iodine, and the raising thereby of the level of iodine-metabolism to a point represented by 85% of iodine per litre of urine, did actually counteract the goitrogenous influence of the insanitary conditions under which certain of the animals lived. From which it would seem to follow that more iodine is needed by the thyroid gland, or less is available for its needs, under conditions which involve contamination of the intestinal tract. It is necessary to add that the degree of insanitation and the composition of the experimental diet were probably not without influence in determining the size of the thyroid enlargements. Temperature and atmospheric conditions during the summer months at Coonoor cause the litter accumulating in the 'dirty cages' to dry rapidly and for this reason the degree of insanitation in these experiments was not extreme. The experimental diet was composed entirely of vegetable and mineral matter. Had it contained a high proportion of animal protein the thyroid enlargements might have been greater, since Burget(14) has observed that unhygienic conditions, in association with a high-protein diet, bring about a greater degree of thyroid enlargement than either factor alone.

When the animals were kept under conditions of meticulous cleanliness, thyroid enlargement did not occur. It follows, therefore, that a level of iodine-metabolism represented by 33% of iodine per litre of urine sufficed for the needs of the thyroid gland of rats fed on this particular diet when they were living under hygienic conditions. This result is in conformity with previous experimental work in this laboratory whereby it was shown that in pigeons fed on a diet of mixed grains meticulous attention to cleanliness prevented a *certain type* of thyroid enlargement (*hypertrophic goitre*) almost as certainly as the additional provision of iodine in the food(8). The application of this principle to the prevention of goitre in the human subject has been attended with success no less striking than that following the prophylactic use of iodine(15).

The results of the two experiments differed in one regard—the effect of the administration of iodized water to animals living in 'clean cages'. In the first experiment the additional iodine, appeared to cause the thyroid gland to be smaller and in the second to be larger. In neither experiment was the difference in size of the glands of animals receiving the iodized water and of those not receiving it very great. Nevertheless, the result cannot be put aside as negligible. The iodized water used in the second experiment was ten times the strength of that used in the first, and whereas the level of iodine-metabolism was raised in the latter from 33% to 85% per litre of urine it was raised in the former from 33% to 559%. In the second experiment this high level of iodine-metabolism appears to have

exercised a stimulating effect on the thyroid gland. If this be so the observation is in conformity with the results of previous experiments carried out in this laboratory (16) and with the observations of Loeb (17) and Rabmovitch (18). Thus in pigeons living under clean conditions the effect of adding iodine to their drinking water was to cause an enlargement of the thyroid gland which though slight was, statistically speaking, of very definite significance (16).

While these experiments show (*vide* 'statistical examination of the experimental data') that young rats living under hygienic conditions and fed on this deficient diet did not develop thyroid enlargement within a period of 64 days though their level of iodine-metabolism was relatively low, it is to be remembered that there is one type of goitre—the *lymph adenoid* (19, 20)—which can arise in deficiently-fed rats despite the most perfect sanitation and despite the adequate ingestion of iodine. It is necessary therefore, to have a clear idea as to the type of thyroid enlargement which was caused in the present experiments by unsanitary conditions of life and prevented by the additional provision of iodine in the food.

### Histological appearances of the thyroids

The thyroids available for histological study were (a) 12 glands from rats which had lived under hygienic conditions ('clean cages'), and (b) 12 glands from rats which had lived under unhygienic conditions ('dirty cages'). In both categories 6 animals had received the experimental diet with iodized water (0.1 mg. of iodine to the litre) and 6 the experimental diet without additional iodine.

There were two questions to which answers were sought from a histological study of these glands. (1) What effect was produced on the structural features of the thyroid gland by a relatively low level of iodine-metabolism (33γ per litre of urine) when the factor of sanitation was excluded? (2) What effect was produced on the structural features of the thyroid gland by unsanitary conditions of life in rats whose level of iodine metabolism was represented by a urinary excretion of 33γ of iodine per litre? The answer to the first question is that no appreciable effect was produced on the gland by an iodine-metabolism of this level when the animals were living under hygienic conditions. The answer to the second is that the thyroid enlargement which resulted from 'dirt' was a compensatory hypertrophy of the organ.

Representative sections—both in low and high power magnification—of the thyroids from rats which had lived *under hygienic conditions*, with and without the additional provision of iodine in their diet, are shown in Plates LXIV, LXV and LXVI, figs 4 to 15. At no stage of the experiment did the thyroids of rats receiving no additional iodine present histological appearances that were not also present in the thyroids of rats receiving additional iodine. On the 35th day of the experiment the major part of the gland, in both groups, was engaged in active secretion, there being relatively few areas—and these at the periphery of the gland—engaged in colloid storage (Plate LXIV, figs 4 and 6). Active secretion was equally well marked in the two groups (Plate LXIV, figs 5 and 7). It was

hygienic conditions of life under which the animals lived. These factors were (a) the relatively low level of iodine-metabolism attained by the rats consuming the diet, (b) the susceptibility of the intestinal tract to infection consequent on the vitamin deficiencies of the diet, and (c) the contamination of the food by the excreta of the animals living under unhygienic conditions of life. Of these three factors the first did not of itself cause thyroid hypertrophy, though it permitted of the goitrogenous action of the third. Nor did the second lead to thyroid enlargement in the absence of the third. The third factor—unhygienic conditions of life—was thus the dominant one. It was the source of a *positive* goitre-producing agency which was counteracted by the administration of iodine.

In connexion with the general effects of unhygienic conditions of life on the animal organism an interesting result has emerged from the statistical analyses of the experimental data provided by the second experiment. There was a significant association not only of big thyroids but of big spleens with unhygienic conditions of life. But whereas the administration of iodine prevented the thyroid enlargement it had no effect on the splenic enlargement. Further, there was a significant tendency for animals living under hygienic conditions of life, and receiving additional iodine, to have small livers.

### Summary.

Young rats were fed on an experimental diet consisting of oatmeal, patent flour, linseed meal, calcium phosphate and sodium chloride. This diet besides being low in iodine was markedly deficient in vitamin A.

The concentration of iodine in the urine was taken as an index of the level of iodine-metabolism.

The experimental diet admitted of a level of iodine-metabolism represented by 33γ of iodine per litre of urine.

This level of iodine-metabolism was similar to that of goitrous persons in certain parts of the world.

It was not associated with thyroid enlargement within the 60–64 days of the experiments, when the rats were kept under conditions of meticulous cleanliness. It was associated with thyroid enlargement, within the same period, when the rats were kept under unhygienic conditions of life.

The type of thyroid enlargement caused by the unhygienic conditions of life was a simple hypertrophy of the gland (*hypertrophic goitre*).

When the level of iodine-metabolism was raised to a point represented by 85γ of iodine per litre of urine the hypertrophic goitre caused by unhygienic conditions was prevented. It was likewise prevented when the level of iodine-metabolism was raised to a point represented by 559γ per litre of urine.

An iodine-metabolism of this high level (559γ per litre of urine), while preventing the thyroid enlargement caused by unhygienic conditions of life, appeared to exercise a stimulating action on the thyroid gland of rats living under hygienic conditions.

Apart from its relatively low content of iodine the experimental diet had faults capable of inducing pathological changes in the thyroid gland

It is pointed out that while *hypertrophic goitre* did not arise in deficiently-fed rats living under hygienic conditions although their level of iodine-metabolism was relatively low there is another type of goitre—the *lymph adenoid*—which does arise in deficiently-fed rats despite conditions of perfect sanitation and despite the adequate ingestion of iodine

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#### EXPLANATION OF PLATE LXIV

- Fig 4 Low power view of thyroid lobe at widest part showing gland, for the most part, engaged in active secretion. A few colloid follicles are scattered around the periphery of the lobe. From a rat living under hygienic conditions (‘clean cages’) and fed on the experimental diet for 35 days *without iodized water*
- „ 5 High power view of actively secreting area from the gland shown in Fig 4
- „ 6 Low power view of thyroid lobe at widest part showing gland, for the most part, engaged in active secretion. A few colloid follicles are scattered around the periphery of the lobe. From a rat living under hygienic conditions (‘clean cages’) and fed on the experimental diet for 35 days *with iodized water*
- „ 7 High power view of actively secreting area from the gland shown in Fig 6



FIG 4

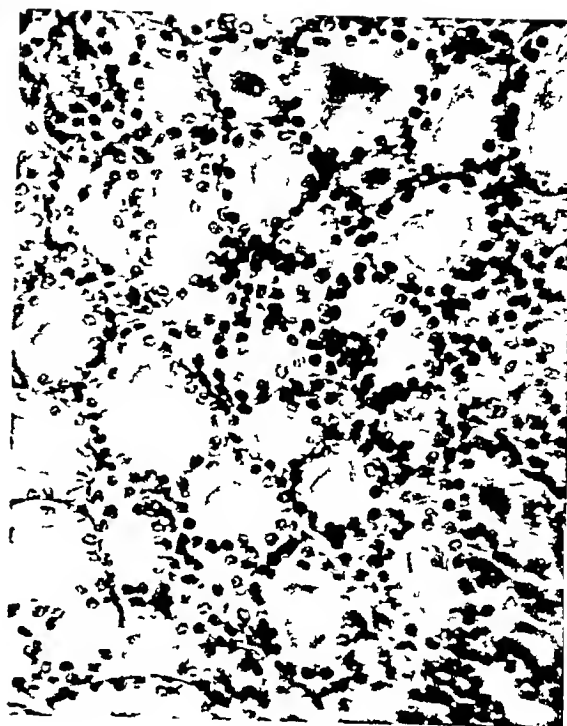


FIG 5

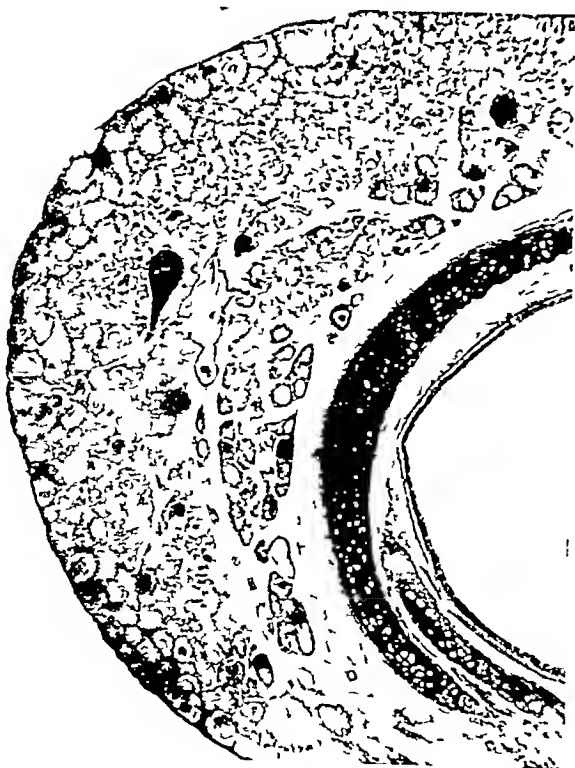


FIG 6



FIG 7





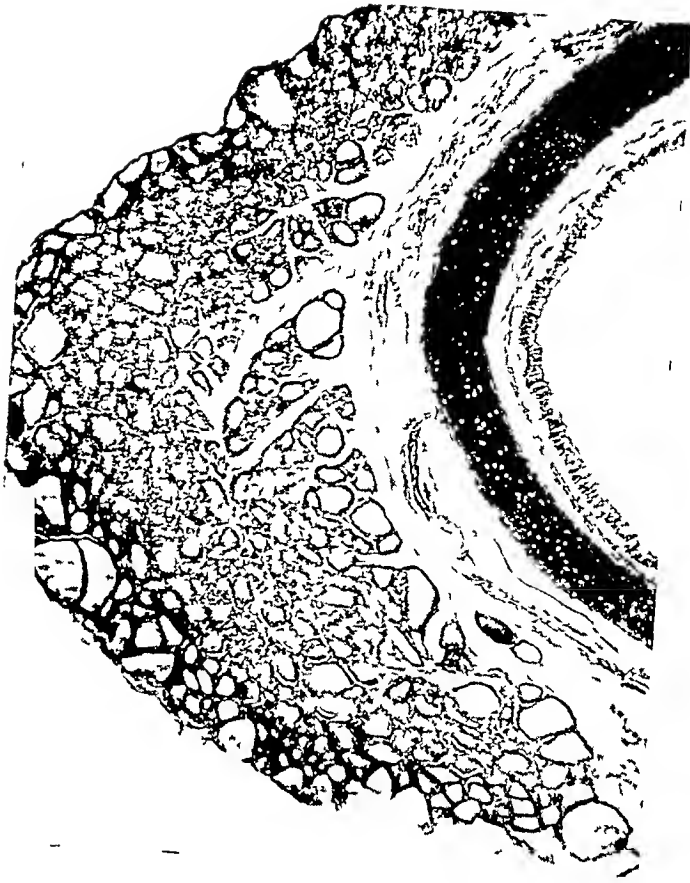


FIG 8

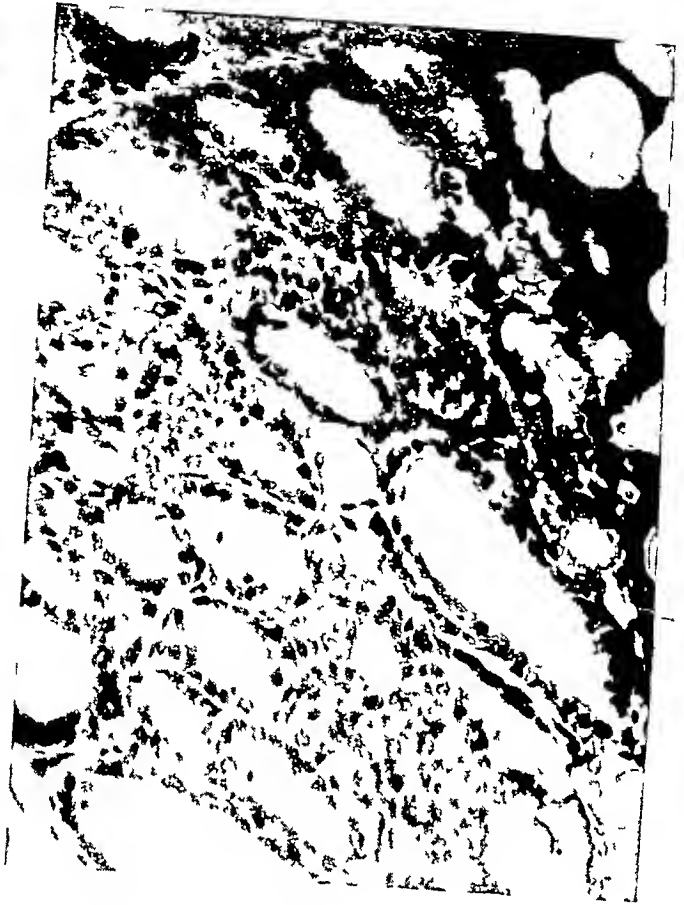


FIG 9.



FIG 10



FIG 11

# EXPLANATION OF PLATE LXV

- Fig 8 Low power view of thyroid lobe at widest part showing more equal distribution of colloid storage follicles and secretion vesicles than on the 35th day From a rat living under hygienic conditions ('clean cages') and fed on the experimental diet for 49 days *without iodized water*
- „ 9 High power view of gland shown in Fig 8
- „ 10 Low power view of thyroid lobe at widest part showing more equal distribution of colloid storage follicles and secretion vesicles than on the 35th day From a rat living under hygienic conditions ('clean cages') and fed on the experimental diet for 49 days *with iodized water*
- „ 11 High power view of gland shown in Fig 10

#### EXPLANATION OF PLATE LXVI

- Fig 12 Low power view of thyroid lobe at widest part showing colloid storage somewhat more in evidence than active secretion From a rat living under hygienic conditions ( ' clean cages ' ) and fed on the experimental diet for 64 *days without iodized water*
- „ 13 High power view of gland shown in Fig 12 some injection of the periacinar capillaries
- „ 14 Low power view of thyroid lobe at widest part showing predominance of colloid storage follicles From a rat living under hygienic conditions ( ' clean cages ' ) and fed on the experimental diet for 64 *days with iodized water*
- „ 15 High power view of gland shown in Fig 14.



Fig 12

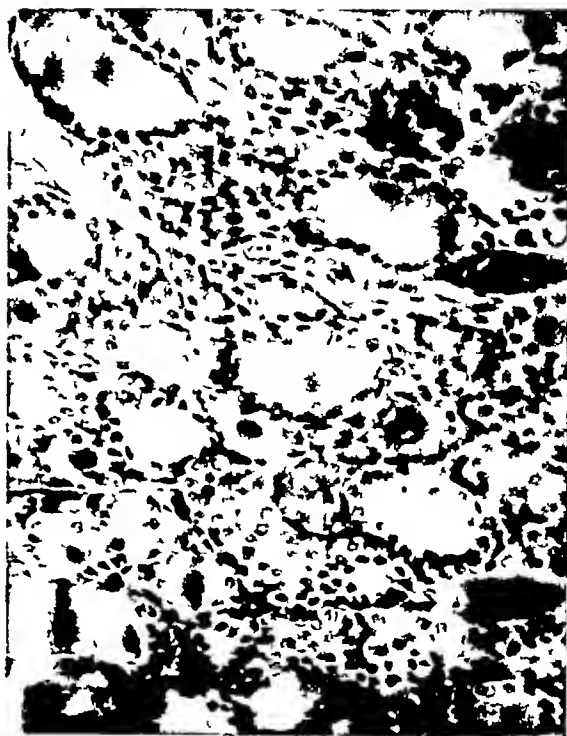


Fig 13







EXPLANATION OF PLATE LXVII

- Fig 16    Posterior view of thyroid lobe of rat living *under unhygienic conditions* ('dirty cages') and fed on the experimental diet for 64 days *without iodized water*. Note lateral and backward growth but otherwise normal appearance

#### EXPLANATION OF PLATE LXVIII

- Fig 17. High power view of actively secreting thyroid gland from rat living *under unhygienic conditions* ('dirty cages') for 35 days and fed on the experimental diet *without iodized water*
- , 18 A similar view of the thyroid gland on the 64th day showing colloid follicles more in evidence than at an earlier stage of the experiment From a rat living under unhygienic conditions ('dirty cages') and receiving *no iodized water*
- „ 19. High power view of actively secreting thyroid gland from a rat living under *unhygienic conditions* ('dirty cages') for 35 days and fed on the experimental diet *with iodized water*
- „ 20 A similar view of the thyroid gland on the 64th day showing secretory activity still predominating, also injection of peri-acinar capillaries From a rat living under unhygienic conditions ('dirty cages') and *receiving iodized water*.

PLATE LXVIII

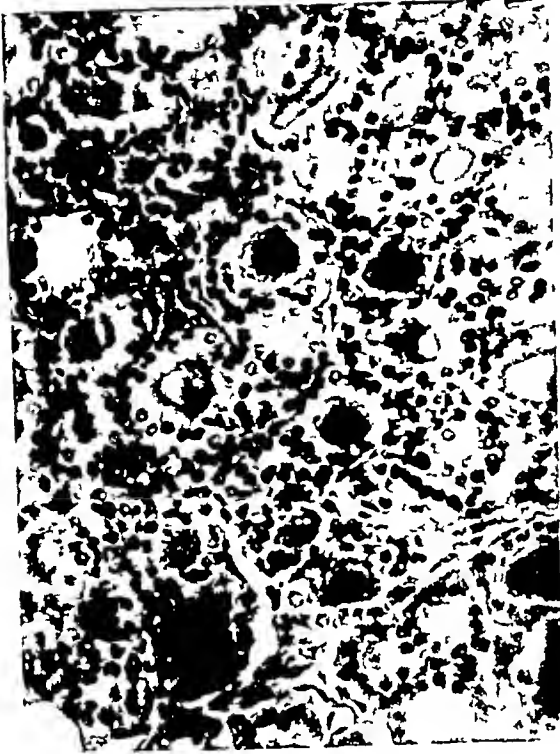


FIG 17



FIG 18

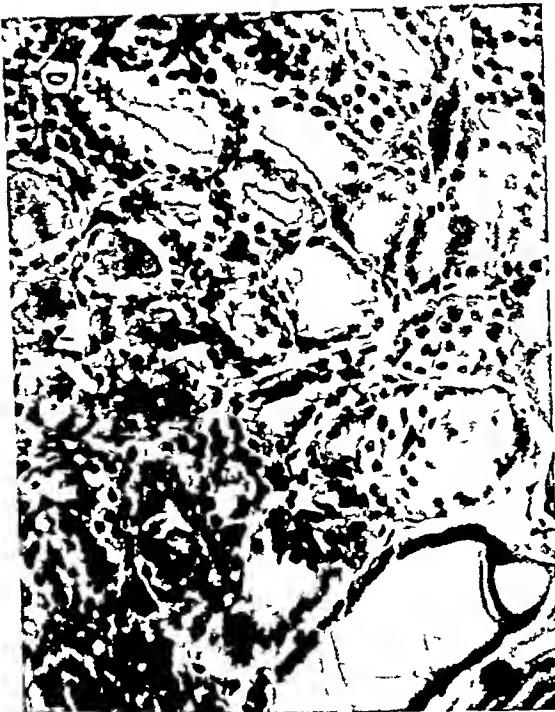


FIG 19.



FIG. 20



## APPENDIX

### A STATISTICAL EXAMINATION OF THE EXPERIMENTAL DATA

#### BY

MAJOR CLIVE NEWCOMB, D M , F I C , I M S

As weights of organs are correlated with body-weights, and as in these experiments the body-weights show large variations—in experiment II significant variations between batches—it is necessary to eliminate this correlation before comparing the weights of the organs

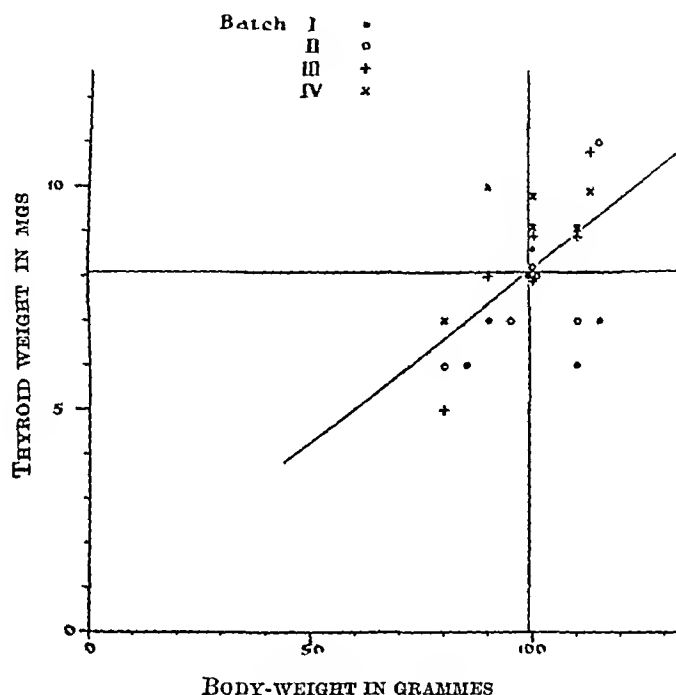
In Donaldson's book *The Rat*(1) the regressions of various organ-weights on body-weights are given. His figures are, however, for a different breed of rat. This is shown by a comparison of the normal body-weight—age curve of the rats used in this laboratory with his. Moreover, Donaldson's figures are for stock rats fed on a well-balanced and adequate diet, the diet used in these experiments being markedly deficient in vitamins, may well have influenced the relations between the size of the organs and the body-weights. The weights of some of the organs in these experiments do not correspond at all to the weights given by Donaldson. The weights of the thyroid glands of our rats are all very much below Donaldson's line for his normal thyroids, so that if one of our rats had a thyroid which according to Donaldson was normal for its body-weight we should consider it as having a goitre. On the other hand the spleens in our experiments are all much bigger than those given by Donaldson. This, however, may be due to the method of killing the rats, as McRoberts(2) has shown with our rats that this greatly affects the size of the spleen. The method of killing being the same throughout these experiments does not, however, affect the comparison of the different batches.

What can be got from Donaldson's figures is this: that within the range of body-weight in these experiments (45—139 grammes) the regressions of the weights of all organs on body-weights are very nearly straight lines.

If the weights of the organs are expressed per 100 grammes of body-weight and the ratios for any organ compared, the assumption is made that the regression line is not only straight but that it cuts the axes at their origin, or, put another way, that the organ has no weight in a rat of no weight. In general the regression lines for the weights of the various organs on body-weights do not tend to cut the axes at their origin.

The data for the first experiment, and some statistics calculated from them, are given in Table I. The thyroid-weights, plotted against body-weights, are shown in Diagram 1 and on this diagram the regression line for the data of this experiment is also given.

DIAGRAM 1.



In order to compare the effect of iodine and of insanitary conditions of life on the thyroid weights, the thyroids have been classed into small and big according as they lie below or above the regression line—those falling very close to the line being omitted. The associations of small and big thyroids with the conditions as to dirt and iodine are shown in Table II. To test the significance of these associations the chi squareds ( $\chi^2$ ) have been calculated(3). Their values are given in column 6 and the corresponding percentage probabilities in column 7. The figures in column 7 indicate the number of times in 100 trials that a result as divergent as the one found might be expected to occur if there were in reality no association between the factors concerned. In view of the paucity of the data the calculation of these probabilities is not very satisfactory but they give some indication of the reliance to be placed on the results.

TABLE I

*Showing the body weights and weights of the thyroid in animals from the first experiment*

Batch	Condition	Body weight in grammes	Thyroid weight in mg	Sex
1	Clean cages with iodine	110	6	M
		85	6	F
		115	7	M
		100	8.6	F
		100	8	M
		90	7	F
	Mean	100	7.1	
2	Dirty cages with iodine	110	7	M
		80	6	F
		115	11	M
		100	8.2	F
		100	8	M
		95	7	F
	Mean	100	7.9	
3	Clean cages without iodine	110	9	M
		80	5	F
		113	10.8	M
		100	8.0	F
		100	9	M
		90	8	F
	Mean	99	8.3	
4	Dirty cages without iodine	110	9	M
		80	7	F
		113	9.9	M
		100	9.8	F
		100	9	M
		90	10	F
	Mean	99	9.1	

Mean body weight

99.4 s.d. 11.0

Mean thyroid weight

. 8.10 s.d. 1.55

Correlation of thyroid weight and body weight + 0.56

Regression of thyroid weight on body weight  $Y = 0.079 X + 0.29$



TABLE II

## FIRST EXPERIMENT

*Showing the associations of small and big thyroids with the conditions of treatment*

1	2	3	4	5	6	7
Batches	Conditions	THYROIDES			$\chi^2$	P per cent
		Small	Big	TOTALS		
I II III IV	Clean with iodine Dirty „ Clean no iodine Dirty „	4 3 1 0	1 1 3 5	5 4 4 5		
		8	10	18		
I and II III and IV	Iodine No iodine	7 1	2 8	9 9		
		8	10	18	8.1	2
I and III II and IV	Clean Dirty	5 3	4 6	9 9		
		8	10	18	0.9	34
I III	Iodine } Amongst those in No iodine } clean cages	4 1	1 3	5 4		
		5	4	9	3.7	10
II IV	Iodine } Amongst those in No iodine } dirty cages	3 0	1 5	4 5		
		3	6	9	5.6	2
I II	Clean } Amongst those getting Dirty } iodine	4 3	1 1	5 4		
		7	2	9		
III IV	Clean } Amongst those not Dirty } getting iodine	1 0	3 5	4 5		
		1	8	9	1.4	24

The conclusions to be drawn from the first experiment are as follows —

- 1 The administration of iodine in the form of iodized water was on the whole associated with small thyroids ( $P=2$ ) This association is more marked amongst rats living under unhygienic conditions ( $P=2$ ) and is of doubtful significance amongst those living under hygienic conditions ( $P=10$ )
- 2 The association of big thyroids and unhygienic conditions is in no case significant but is most marked amongst those not receiving iodized water ( $P=24$ ) This suggests that subsequent experiments might show that iodine prevents an enlargement of the thyroid due to dirt

In view of the small numbers of observations this experiment, if it stood alone, would not admit of sure conclusions The results of this experiment were, however, for the most part confirmed by those of the second

The experimental data of the second experiment are given in Table III In this experiment other organs besides the thyroid were weighed as it appeared possible that a statistical examination of the data relating to these organs might serve to indicate whether the effects of the two influences under consideration—iodine and unsanitary conditions of life—were peculiar to the thyroid gland In this experiment the rats were all killed at the same age The average body-weights of the four batches differed, these differences were also examined as to the effects of iodine and of 'dirt' on the body-weights

Certain statistics calculated from the data in Table III are shown in Table IX Those relating to body-weights, thyroids and livers have been calculated without grouping, and those relating to other organs by means of small correlation tables

TABLE III

*Showing the body-weights and weights of organs in animals in the second experiment*

	Body weight grms	Thyroid weight mg	Adrenal weight mg	Heart weight grms	Liver weight grms	Kidney weight grms	Spleen weight grms
I Clean with iodine	50		60	0 27	2 32	0 71	0 18
	83	7 8	30	0 36	4 23	0 90	0 47
	100	9 0	20	0 41	3 66	0 87	0 48
	78	9 4	26	0 37	3 27	0 82	0 44
	65	8 4	20	0 80	2 90	0 66	0 33
	90	9 2	30	0 39	4 02	0 89	0 49
	70	6 6	30	0 34	3 35	0 78	0 32
	73	7 0	20	0 35	2 93	0 84	0 47
	63		20	0 35	3 21	0 76	0 43

TABLE III—*concl'd*

	Body weight grms	Thyroid weight mg	Adrenal weight mg	Heart weight grms	Liver weight grms	Kidney weight grms	Spleen weight grms
2 Dirty with iodine	76	88		0 34	3 94	0 86	0 54
	83	88	20	0 35	3 53	0 83	0 92
	139	108	22	0 55	7 28	1 26	1 33
	106	86	21	0 40	5 11	0 90	0 62
	80	68	20	0 34	3 71	0 72	0 69
	79	92	20	0 43	3 67	0 78	0 50
	68	80	28	0 42	3 46	0 68	0 51
	86	70	19	0 39	4 35	0 83	0 77
	66	52	50	0 34	4 02	0 87	0 36
3 Clean without iodine	54	52	20	0 28	2 76	0 74	0 60
	92	90	21	0 39	4 18	0 49	0 49
	106	80	20	0 44	5 12	1 18	0 56
	93	70	30	0 37	5 25	0 97	0 48
	70	64	19	0 32	3 86	0 83	0 30
	55	48	20	0 27	2 52	0 72	0 23
	45	50	27	0 24	2 44	0 62	0 16
	72	84	20	0 31	3 75		0 39
	46		32	0 34	3 50	0 64	0 17
	55		40	0 29	2 68	0 77	0 21
	53		20	0 29	2 65	0 75	0 17
4 Dirty without iodine	58	54	30	0 27	2 69	0 79	0 37
	78	86	30	0 46	3 60	0 84	0 55
	87	92	30	0 44	3 95	0 85	0 61
	87	108	21	0 41	3 27	0 83	0 58
	64	72	11	0 32	2 95	0 75	0 65
	87	98	29	0 35	3 54	0 84	0 52
	88	96	20	0 36	4 70	0 87	0 57
	93	102	20	0 38	4 78	0 97	0 53
	50		30	0 32	2 73	0 71	0 62
	65		50	0 32	4 17	0 82	0 34

The statistics for the last may, therefore, have small errors due to grouping. To be sure that the errors are really small the partial standard deviations have been checked by comparing the value calculated from the coefficient of correlation with the value calculated by summing the squares of the deviations from the regression lines and in no case is the difference of the two values considerable. The correlation coefficients have been calculated without Sheppard's correction.

TABLE IV  
*Showing various statistics calculated from the data in Table III*

			Number of obser- vations	Means	Standard deviations
1	Body weights	grammes	39	75.7	19.2
2	Thyroids	mg	32	7.97	1.66
3	Adrenals	"	38	26.3	9.85
4	Hearts	grammes	39	0.35	0.062
5	Livers	"	39	3.69	0.954
6	Kidneys	"	38	0.82	0.16
7	Spleens	"	39	0.49	0.21
Body weights corresponding to the 32 thyroids			32	80.4	17.9

Correlation	coefficients	Standard deviations	
$\gamma_{12}$	+0.71	$\sigma_{2.1}$	1.17
$\gamma_{13}$	-0.30	$\sigma_{3.1}$	9.4
$\gamma_{14}$	+0.84	$\sigma_{4.1}$	0.033
$\gamma_{15}$	+0.87	$\sigma_{5.1}$	0.47
$\gamma_{16}$	+0.69	$\sigma_{6.1}$	0.10
$\gamma_{17}$	+0.72	$\sigma_{7.1}$	0.15
$\gamma_{..}$	+0.49		
$\gamma_{..1}$	-0.04		
$\gamma_{17}$	+0.59		
$\gamma_{6.1}$	-0.10		

#### BODY-WEIGHTS

The median body-weight of the 39 rats in this experiment is 75 grammes. Grouping the animals as small and big, according as their body-weights are below or

above 75 grammes, the associations of treatment and body-weight are shown in Table V

TABLE V  
SECOND EXPERIMENT

*Showing the association of small and big body-weights with the treatment*

1	2	3	4	5	6	7		
Batches	Conditions	THYROIDES			$\chi^2$	P per cent		
		Small	Big	TOTALS				
I	Clean with iodine	5	4	9				
II	Dirty "	2	7	9				
III	Clean without iodine	8	3	11				
IV	Dirty " "	4	6	10				
		19	20	39	5.51	6		
I and II III and IV	Iodine No iodine	7 12	11 9	18 21				
		19	20	39	1.29	26		
I and III II and IV	Clean Dirty	13 6	7 13	20 19				
		19	20	39	4.36	4		
I III	Iodine No iodine	Amongst those in clean cages		5 8	4 3	9 11		
		13	7	20	0.64	41		
II IV	Iodine No iodine	Amongst those in dirty cages		2 4	7 6	9 10		
		6	13	19	0.69	39		
I II	Clean Dirty	Amongst those getting iodine		5 2	4 7	9 9		
		7	11	18	2.11	15		
III IV	Clean Dirty	Amongst those not getting iodine		8 4	3 6	11 10		
		12	9	21	2.29	13		

The conclusions to be drawn from Table V are —

- 1 There is no demonstrable effect of iodine on body-weight
- 2 The rats in dirty cages were heavier than those in clean cages ( $P=4$ ) and this was so irrespective of the addition or withholding of iodine ( $P=15$  and  $P=13$ )

A similar result is got if the mean body-weights of the batches are compared, and the differences tested for significance as suggested in Newcomb(4)

#### WEIGHTS OF ORGANS

The weights of all the organs were positively correlated with the body-weights (*vide* Table IV), except the weights of the adrenal glands. In this experiment the correlation of the last comes out negative but the figure ( $-0.295$ ) is too small to be definitely significant and is due almost entirely to two outstanding observations (1 and 2 in Table III). The adrenal glands are omitted in the subsequent analysis.

TABLE VI

*Showing the mean differences in the weights of the various organs in the four batches from the regression lines on body-weights*

	BATCH I	BATCH II	BATCH III	BATCH IV
	Clean with iodine	Dirty with iodine	Clean without iodine	Dirty without iodine
<i>Thyroids</i>				
Numbers	7	9	8	8
Means	+0.200	-0.233	-0.850	+0.875
St errors of means	0.442	0.391	0.414	0.414
<i>Hearts</i>				
Numbers	9	9	11	10
Means	+0.001	+0.013	-0.007	+0.012
St errors of means	0.011	0.011	0.010	0.011
<i>Livers</i>				
Numbers	9	9	11	10
Means	-0.293	+0.164	+0.248	-0.026
St errors of means	0.157	0.157	0.142	0.149
<i>Kidneys</i>				
Numbers	9	9	10	10
Means	-0.003	-0.014	+0.007	+0.014
St errors of means	0.033	0.033	0.032	0.032
<i>Spleens</i>				
Numbers	9	9	11	10
Means	-0.080	+0.120	-0.081	+0.047
St errors of means	0.049	0.049	0.044	0.046

In the second experiment a slightly different procedure has been adopted to compare the weights of the various organs in the four batches while getting rid of the effect of body-weight. The procedure is —

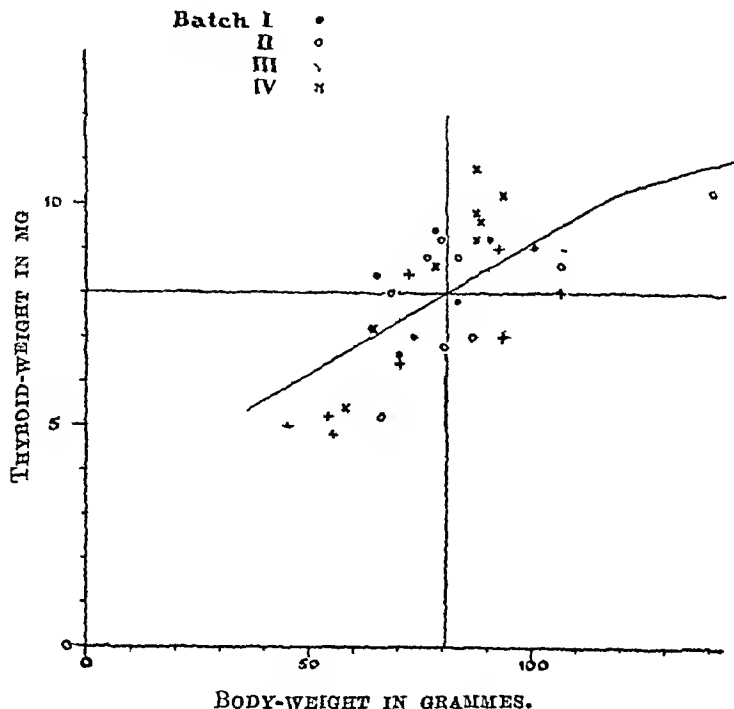
- 1 On the supposition that the weights of the organs are uninfluenced by the conditions as to iodine or dirt, the weight which each organ would be expected to have from the body-weight of the animal has been calculated from the found regression of the various organ-weights on body-weights
  - 2 The differences between the expected weights, and the actual weights have been tabulated
  - 3 The mean differences from expectation in the four batches have been calculated and are given in Table VI
- To judge of the significance of these differences —
- 4 The partial standard deviations of the various organs about the regression lines have been calculated and are given in Table IV
  - 5 Using these standard deviations the standard errors of the means of the differences have been tabulated in Table VI

There are too few observations for the satisfactory calculation of a standard deviation of the mean differences from expectation for each batch separately

#### THYROIDS

The regression of thyroid-weights on body-weights for the second experiment is shown in Diagram 2

DIAGRAM 2



The significances of the differences between the means of the differences from expectation in the four batches are shown in Table VII

Comparing first those getting and not getting iodine if all those getting iodine (I and II) are compared with all those not getting iodine (III and IV), the difference ( $-0.029$ ) is slight and insignificant, and this is so because the effect of iodine is apparently different under clean conditions and under dirty conditions

Comparing those in clean cages with each other (I—III) the giving of iodine is associated with *larger* thyroids ( $P=12$ ). In this experiment there is apparently a stimulating effect of iodine on the thyroid gland in the animals living in clean cages

Comparing those in dirty cages with each other (II and IV) the giving of iodine is associated with *smaller* thyroids ( $P=9$ )

Comparing next the effect of dirty and clean cages if all those in clean cages (I and III) are compared with all those in dirty cages (II and IV), those in dirty cages had larger thyroids though the difference ( $-0.646$ ) is not very significant ( $P=13$ ), the want of good significance again being due to the seemingly stimulating effect of iodine under clean conditions. Comparing those getting iodine with each other (I and II) the effect of dirt is insignificant. Comparing on the other hand those not getting iodine with each other (III and IV) those in dirty cages had definitely larger thyroids ( $+1.725$   $P=2$ )

TABLE VII

*Showing the significance of the differences between thyroid-weights in the four batches*

Batches	Numbers	Means	Standard errors	Diff st errors	P per cent
I	7	+0.200	0.44	0.45	8 7
II	9	-0.233	0.39	0.59	
III	8	-0.850	0.41	2.05	
IV	8	+0.875	0.41	2.11	
I and II	16	-0.017	0.29	0.07	
III and IV	16	+0.012	0.29		
	Difference	-0.029	0.42		
I and III	15	-0.325	0.30	1.55	13
II and IV	17	+0.321	0.28		
	Difference	-0.646	0.42		
I—II		+0.433	0.59	0.73	12
I—III		+1.050	0.60	1.75	
I—IV		-0.675	0.60	1.12	
II—III		-0.617	0.57	1.08	
II—IV		-1.108	0.57	1.95	9
III—IV		-1.725	0.58	2.98	2



## HEARTS AND KIDNEYS

Comparing the effect of iodine and no iodine and of dirty and clean cages in a similar way to that used for thyroids, no significant associations are brought to light between either of the conditions and heart-weights or kidney-weights

## LIVERS

The comparisons of the liver-weights, on the same plan as for the thyroid-weights, is shown in Table VIII. Only two differences are statistically at all significant, viz, I—II and I—III, these indicate that amongst rats living in clean cages the association between iodine and small livers is significant ( $P=2$ ) and amongst those getting iodine the association between clean cages and small livers is significant ( $P=7$ ). The results are compatible with the theory that giving iodine reduces the size of the liver and that dirt destroys or neutralizes the effect of iodine

TABLE VIII

*Showing the significances of the differences between liver-weights in the four batches*

Batches	Numbers	Mean diff	Standard errors	Diff st errors	P per cent
I	9	—0.293	0.157		
II	9	+0.164	0.157		
III	11	+0.248	0.142		
IV	10	—0.026	0.149		
I and II	18	—0.064	0.111		
III and IV	21	+0.111	0.103		
	Difference	—0.175	0.151		
I and III	20	—0.022	0.105		
II and IV	19	+0.069	0.108		
	Difference	—0.091	0.151		
I—II		—0.457	0.222	2.06	7
I—III		—0.545	0.212	2.67	2
I—IV		—0.267	0.216		
II—III		—0.084	0.212		
II—IV		+0.190	0.216		
III—IV		+0.274	0.206		

## SPLEENS

There is no demonstrable association between spleen-weights and the giving of iodine (*vide* Table IX), but there is a significant association of big spleens with dirty

conditions of life, both on the whole ( $P=0.5$ ) and amongst those with iodine ( $P=2$ ) and without iodine ( $P=8$ )

Big spleens are associated with big thyroids (Table IV  $\gamma_{27}=+0.49$ ) but this association is all due to the association of both with body-weight (*ibid*  $\gamma_{27.1}=-0.01$ ). In the same way big spleens are associated with big livers (*ibid*  $\gamma_{57}=+0.59$ ) but here again the association is due to the association of both with body weight (*ibid*  $\gamma_{57}=-0.10$ ). The numbers of observations are insufficient for carrying this investigation on to separate batches.

TABLE IX

*Showing the significance of the differences between spleen weights in the four batches*

Batches	Numbers	Mean diff	Standard errors	Diff st errors	P per cent
I	9	-0.080	0.049	1.63	14
II	9	+0.120	0.049	2.46	4
III	11	-0.081	0.041	1.84	10
IV	10	+0.047	0.046	1.02	
I and II	18	+0.020	0.034		
III and IV	21	-0.017	0.032		
	Difference	+0.037	0.047		
I and III	20	-0.081	0.033		
II and IV	19	+0.083	0.034		
	Difference	-0.164	0.047	3.49	$\frac{1}{2}$
I—II		-0.200	0.070	2.86	2
I—III		+0.001	0.066		
I—IV		-0.127	0.068	1.87	10
II—III		+0.201	0.066	3.05	1
II—IV		+0.073	0.068		
III—IV		-0.128	0.064	2.00	8

## THE TWO EXPERIMENTS COMBINED

The association of small and big thyroids with the conditions as to dirt and iodine in both experiments put together are shown in Table X

TABLE X

## BOTH EXPERIMENTS COMBINED

*Showing the association of big and small thyroids with dirty cages and iodine*

Batches	Conditions	THYROIDS			$\chi^2$	P per cent
		Small	Big	TOTALS		
I II III IV	Clean with iodine Dirty       " Clean without iodine Dirty       "	9 9 8 2	4 6 6 12	13 15 14 14	9.96	1.8
		28	28	56		
I and III II and IV	Clean Dirty	17 11	10 18	27 29	3.13	7.5
		28	28	56		
I and II III and IV	Iodine No iodine	18 10	10 18	28 28	4.6	3.3
		28	28	56		
I II	Clean } Amongst those getting Dirty } iodine	9 9	4 6	13 15	No association	
		18	10	28		
III IV	Clean } Amongst those not Dirty } getting iodine	8 2	6 12	14 14	5.6	1.8
		10	18	28		
I III	Iodine } Amongst those in No iodine } clean cages	9 8	4 6	13 14	No association	
		17	10	27		
II IV	Iodine } Amongst those in No iodine } dirty cages	9 2	6 12	15 14	6.42	1.1
		11	18	29		

The conclusions to be drawn from this Table confirm the previous conclusions and are —

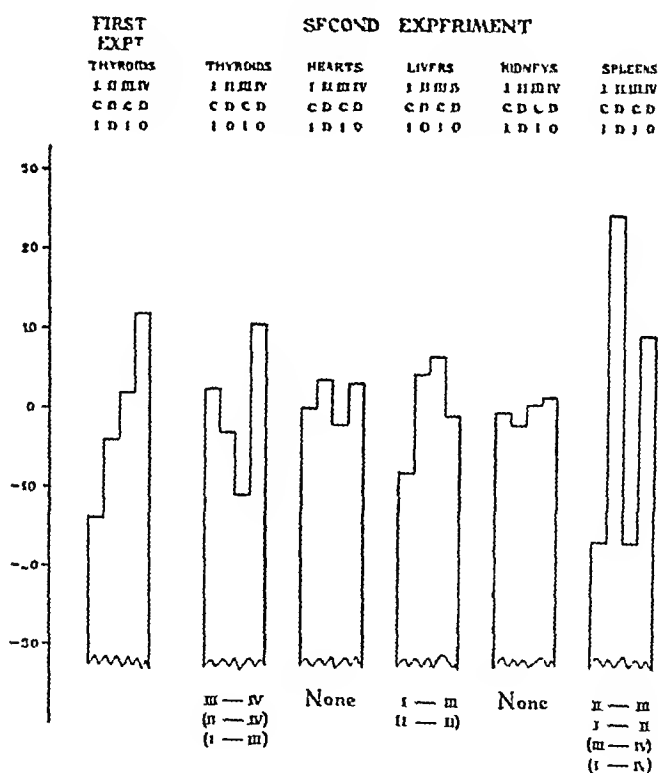
- 1 Big thyroids are associated with unhygienic conditions of life ( $P=7.5$ ), and this association is only definitely shown by the rats not receiving iodized water ( $P=1.8$ ). Amongst the rats getting iodized water this association is absent.
- 2 Small thyroids are associated with the giving of iodized water ( $P=3.3$ ) and this association is only definitely shown by the rats living under unhygienic conditions ( $P=1.1$ ) and is absent amongst those in clean cages.

Translating associations into causes

Dirt causes enlargement of the thyroid gland and the administration of iodized water prevents this enlargement.

The results of the two experiments are summarized, and perhaps made clearer, in Diagram 3.

DIAGRAM 3



In this diagram the mean differences of the weights of organs in the four batches from the weights which would be expected from the body-weights on the supposition that the conditions as to iodine and dirt had no influence, are expressed

as percentages of the mean weight of the organ in question    The conditions are shown at the top of the diagram using symbols —

C=living under hygienic conditions

D=living under unhygienic conditions

I=receiving iodized water

O=not receiving iodized water

The differences which are statistically significant ( $P = < 5$  per cent) are shown at the bottom, those which are only probably significant ( $P = 5-10$  per cent) being enclosed in brackets

### CONCLUSIONS

The statistical analysis of the data given in Tables I and III show them to be compatible with the following conclusions —

- (1) The unhygienic conditions of life under which certain of the rats lived gave rise to enlargement of the thyroid gland
- (2) The provision of additional iodine in the diet prevented this enlargement
- (3) Unhygienic conditions of life also caused enlargement of the spleen, but this enlargement was uninfluenced by the provision of additional iodine
- (4) Rats living under hygienic conditions of life and receiving additional iodine had smaller livers than those living under unhygienic conditions and not receiving additional iodine

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# THE INFLUENCE OF LIME IN FAVOURING THE PRODUCTION OF STONE-IN-THE-BLADDER IN RATS

BY

COLONEL R. MCCARRISON, CIL RHP, MD, FRCP, IMS,  
Director, Nutritional Research I R F A, Pasteur Institute, Coonoor,  
S India

[ Received for publication, October 14, 1929 ]

In order to ascertain whether lime exercised any influence in favouring the production of vesical calculus the following experiments were carried out —

- 1 Twenty-four young rats were fed on an exclusive diet of white flour and distilled water *ad libitum*
- 2 Twelve young rats were fed on a diet consisting of 95 parts of white flour, 5 parts of marmite and distilled water *ad libitum*
- 3 Forty-eight young rats were fed on a diet consisting of 97 parts of white bread, 3 parts of dried yeast and distilled water *ad libitum*
- 4 Twenty-four young rats were fed on a diet consisting of 97 parts of white bread, 3 parts of dried yeast and distilled water *ad libitum*, 5 grams of slaked lime being added to each 20 grammes of the ration

The results of the experiments were as follows —

Diet	Number of animals fed on diet	Number of cases of stone	Percentage incidence of stone
White flour only	24	2	8.3
White flour and marmite	12	1	8.3
White bread and dried yeast	48	8	16.6
White bread, dried yeast and lime	21	9	42.8

These results show that

- (1) An exclusive diet of white flour and distilled water is capable of causing vesical calculus in rats,

(2) The addition of vitamin B, in the form of marmite or dried yeast, to the white flour diet causes no appreciable difference in the incidence of stone,

(3) The further addition of lime causes an increase in the incidence of stone which, statistically speaking, is very significant

These results are to be contrasted with those of a previous experiment(1) in which 24 young rats were fed on a complete diet containing the substances lacking in the diets used in the present experiments suitable protein, fat-soluble vitamins and vitamin C provided in the form of milk, butter and green vegetables. In addition to the complete diet the animals consumed 7 to 10 grams of slaked lime daily during a period of eight and a half months. None of them developed stone. It may be concluded, therefore, that the favouring effect of lime on stone-formation is not exhibited when a sufficiency of suitable proteins, fat-soluble vitamins and vitamin C is present in the food. There is no evidence, from previous experimental work, that either vitamin-free protein or vitamin C exercises any protective action against stone, so far as is at present known this action appears to be confined to vitamin A.

The average calcium-content of bladder stones in rats not receiving lime was found to be 1.2 per cent (dry basis), that of gravel removed from pyonephrosis in a rat which received lime was 38.4 per cent. In the latter the lime was present, for the most part, as carbonate. The average oxalate-content of vesical calculi in rats not receiving lime was 0.8 per cent (dry basis), that of gravel removed from pyonephrosis in a rat which received lime was 9.4 per cent(2).

### CONCLUSION

The ingestion of lime in excess of requirements, by rats fed on a diet deficient in suitable protein, fat-soluble vitamins and vitamin C, exercises an influence which is markedly favourable to stone-formation.

This conclusion is in conformity with the widespread belief that the long continued consumption of hard water is favourable to the formation of stone. It would seem, however, that the excessive ingestion of lime will not exercise any such effect in the presence of a properly constituted diet.

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# THE RELATIVE POTENCY OF CERTAIN CEREAL GRAINS IN FAVOURING THE FORMATION OF STONE IN RATS

BY

COLONEL R McCARRISON, CIL, KHP, MD, FRCP, IMS,  
*Director, Nutritional Research, I R F A, Pasteur Institute, Coonoor,  
S India*

[ Received for publication, October 14, 1929 ]

As is well known, stone-in-the-bladder is much commoner in certain parts of India than in others. It is, in general, most prevalent amongst the wheat-eating races of the north and relatively rare amongst rice-eaters of the south. It seemed possible, therefore, that the different cereals in use in different parts of India might vary in their capacity to favour the formation of stone. This supposition was strengthened by observing that when oatmeal—the main constituent of my original stone-producing diet(1)—was replaced by whole wheat flour (*atta*)(2) there was, statistically speaking, a significant increase in the incidence of stone amongst the experimental animals. Accordingly, an experiment was devised to determine the relative stone-producing potency of four of the chief cereal grains in use in India: wheat (*Triticum vulgare*), millet or ragi (*Eleusine coracana*), rice (*Oryza sativa*), and *cambu* or *bagri* (*Pennisetum typhloideum*). Owing to limitations of space and of the number of rats of the requisite age available for the work it was not possible to deal with more than four cereals at the same time. Other important food grains—maize and *chulam*—had of necessity to be omitted from the investigation.

Each of the four grains formed the basis of an experimental diet, in other respects the four diets had the same composition. This was as follows—

Cereal grain (wheat or millet or rice or <i>cambu</i> )	90 parts
Gingelly (sesame) oil	8 parts
Calcium phosphate	1 part
Sodium chloride ..	1 part
Distilled water	<i>ad libitum</i>

One hundred and twenty-five young rats were used for the experiment: 57 males and 68 females. All were of approximately the same age and body-weight at the outset of the experiment. Each animal was confined in a separate cage under conditions of scrupulous cleanliness. Thirty-five were fed on 'the wheat



diet', 30 on 'the millet diet', 30 on 'the rice diet', and, 30 on 'the *cambu* diet' The experiment was continued for 515 days All the animals fed on 'the wheat diet' had died by the 351st day, all fed on 'the millet diet' had died by the 484th day, all fed on 'the rice diet' by the 212th day, while of those fed on 'the *cambu* diet' 16 were alive on the 515th day, when they were killed So far as capacity to sustain life was concerned *cambu* was the best grain

Although stone-in-the-bladder has been encountered as early as the 33rd day in rats fed on a stone-producing diet(3) this early appearance of the condition is exceptional It is rarely found at post-mortem examination before the twelfth week The period of survival of the animals has, therefore, to be taken into consideration in estimating the relative stone-producing potency of the four cereal grains Of the 35 animals fed on 'the wheat diet' 34 survived for 12 weeks or longer, of the 30 fed on 'the millet diet' 26 survived for 12 weeks or longer, of the 30 fed on 'the rice diet' 22 survived for 12 weeks or longer, while of those fed on 'the *cambu* diet' all survived for 12 weeks or longer

The incidence of stone in the four groups is shown in the following Table —

TABLE I

Diet	Number of animals fed on the diet	Average period of survival days	Number of animals surviving for 12 weeks or longer	Number of animals found to have stone at post mortem	Percentage incidence of stone in animals surviving for 12 weeks or longer	First day on which stone was found at post mortem
Wheat diet	35	235	34	11	31.7	181st
Millet diet	30	263	26	2	7.7	345th
Rice diet	30	114	22	2	9.1	169th
<i>Cambu</i> diet	30	427	30	0	0.0	

In order of stone-producing potency wheat comes first and *cambu* last, millet and rice occupying an intermediate position well below that of wheat So far as the incidence of stone is concerned there is no significant difference between millet and rice but stone made its appearance earlier on 'the rice diet' than on 'the millet diet,' from which it would seem that rice is the more potent of the two in favouring the formation of stone The freedom from stone of the animals fed on 'the *cambu* diet' is as remarkable as the superiority of this grain in sustaining life It had however a peculiar effect not exhibited by the other grains that of causing *Hydrops Testis* a matter dealt with in another paper (page 1109)

In ascending order of stone-producing potency the four grains may thus be grouped as follows —*cambu*, millet, rice and wheat The significantly higher

incidence of vesical calculus in wheat-eating than in rice-eating rats is in conformity with the higher incidence of the disease in wheat-eating than in rice-eating races of India

In a previous paper(2) a contrast was afforded between the stone-producing potency of whole wheat flour (*atta*) and oatmeal. Of 50 rats fed on a diet consisting of 53 parts of oatmeal, 20 parts of linseed meal, 25 parts of cornflour, 1 part of calcium phosphite, 1 part of sodium chloride and distilled water *ad libitum* 4, or 8 per cent developed stone, while of 50 fed on a diet of this composition in which the oatmeal was replaced by whole wheat flour (*atta*) 11 or 22 per cent, developed stone. This difference is statistically significant, whole wheat flour has therefore, a more potent influence than oatmeal in favouring the formation of stone.

It is of interest to contrast the incidence of stone in rats fed on whole wheat flour diets with that in rats fed on white flour or white bread diets, the main deficiency—that of fat-soluble vitamins—being the same in both. For this purpose the following results, obtained in these laboratories, are available —

TABLE II

Diet	Number of animals fed on diet	Number of cases of stone	Percentage incidence of stone
White flour only	24	2	8.3
White flour 87 parts, olive oil 8 parts and marmite 5 parts	12	1	8.3
White flour and marmite (5 per cent)	12	1	8.3
White bread and dried yeast (3 per cent)	48	8	16.6

Taking the white flour and white bread diets together there are 12 cases of stone amongst 96 animals, or an incidence of 12.5 per cent. The highest incidence on any of these diets is 16.6 per cent and this is not significantly different from the others. The lower incidence of stone in rats fed on these white flour or white bread diets, as compared with that in rats fed on whole wheat flour diets is, however, statistically significant, and indicates that whole wheat flour is more potent to favour the formation of vesical calculus than white flour. This fact may have something to do with the diminished incidence of vesical calculus during the past 50 years in European countries(6) a period coinciding with the gradually increasing use of white flour. It would seem that in the manufacture of white flour 'something' favourable to stone formation is partially removed from wheat. It must be emphasized, however, that it is only when certain other factors are present in the food in insufficient quantities that whole wheat is capable of exercising its stone-producing effect. It has been shown in previous papers (4, 5) that rats, fed

on stone-producing diets containing a high proportion either of whole wheat flour or of oatmeal, are afforded complete protection against the malady by the consumption of a sufficient amount of whole milk daily. There are, therefore, protective substances (proteins\* and/or fat-soluble vitamins) in milk whose presence in sufficient quantity in the diet prevents the stone-producing action of some positive factor contained in whole wheat.

It seems probable that the relative stone-producing potency of the cereals examined depends upon some of them requiring more of these protective substances than others. Whole wheat flour, for instance, requiring more than either rice or white flour. But the excellent food—whole wheat flour—ought not to be decried because of its relatively high stone-producing potency, when the fault lies rather in the absence from the diets containing it of a sufficiency of other essential substances. Nevertheless, the evidence so far accumulated points not only to the important influence of negative factors in the genesis of stone but also to that of certain positive factors of which 'something' in whole wheat is one, and lime—as shown in another paper in this issue (page 1101)—is another.

It has recently been objected(6) in connexion with the use of rats for experimental work on stone that these animals are very prone to the disease, and the suggestion has been made that 'it would be a distinct advance if it could be shown that animals normally free from the disease develop stone when kept on a deficient diet'(6). Rats, at least in these laboratories, are normally free from stone. An experience of several thousand post-mortem examinations of these animals has failed to reveal a single case of stone except in those that were deficiently fed. The stock diet consists of whole wheat flour (*atta*) chapattis, sprouted gram, fresh vegetables (carrot, cabbage, etc.) and milk, with a small ration of raw meat occasionally. This diet affords my rats complete protection against vesical and renal calculus.

#### SUMMARY.

1 Cereal grains, when they form the bulk of the diet, vary in their capacity to favour the formation of stone-in-the-bladder in rats. In order of potency they examined range themselves as follows—wheat, oats, rice, millet and *cambu*.

2 A diet containing 90 parts of *cambu* did not cause stone within a period of 515 days, whereas one containing the same amount of whole wheat flour (*atta*) caused stone in 31.7 per cent of the rats fed upon it within a period of 351 days.

3 Millet and rice have approximately the same degree of potency in favouring the formation of stone, both are less potent than oatmeal and much less potent than whole wheat flour.

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\* Since this paper was written further experimentation has shown that vitamin-free proteins do not protect against stone in the absence from the diet of a sufficiency of vitamin A.  
—R. McC, 5-3-30

4 White flour is less potent than whole wheat flour in favouring the formation of stone in rats

5 It is only in the absence from the diet of a sufficiency of certain protective substances (proteins\* and/or fat-soluble vitamins) such as are contained in whole milk that the cereal grains are capable of exercising a stone-producing effect

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| (3) <i>Idem</i> (1928) | <i>Ibid</i> , Vol XV, p 801              |
| (4) <i>Idem</i> (1927) | <i>Ibid</i> , Vol XV, p 485              |
| (5) <i>Idem</i> (1926) | <i>Ibid</i> , Vol XIII, p 817            |
| (6) JOLA (1929)        | 'Stone' London                           |

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\* See footnote on previous page

It will be noted that the animals fed on the *cambu* diet survived, on the average, much longer than those fed on diets having millet, rice or wheat as their basis. It is, therefore, impossible to say whether the *Hydrops testis* was due to some property of, or deficiency in, the *cambu* which was not common to the other cereals, or whether a prolonged period of time was necessary for the development of the condition, the *cambu* diet, probably by reason of a higher content of vitamin A, being the only one of the four diets which admitted of survival of the animals for this period.

The macroscopical appearance of the dropsical testes is shown in Plate LXIX, fig 1, the normal appearance is shown in Plate LXIX, fig 2. *Hydrops testis* was invariably bilateral: the organs were enlarged, yellowish in colour, tense and glistening, the vessels normally winding over their surface being no longer visible. The weight of both testes ranged from 1.39 to 5.60 grammes, or from 16 to 300 per cent above the normal weight of the organs in rats of the same body-weight(2). Their glistening, distended appearance and increased weight were due to distension of the cavity of the tunica vaginalis by pale, amber-coloured fluid having the characters usual to serous effusions.

Microscopically the seminiferous tubules were seen to be widely separated by the serous effusion lying between them (Plate LXX, fig 3). The tubules themselves exhibited varying degrees of degenerative change: some were to all appearance normal (Plate LXX, fig 4) except for slight disruption of their normally compact structure (Plate LXX, figs 5 and 6), others were degenerated in lesser (Plate LXXI, figs 7 and 8) or greater (Plate LXXI, figs 8 and 9) degree. *Hydrops testis* has not previously been encountered in these laboratories nor, so far as I am aware, has a similar condition been recorded in the literature although œdema of the testis has been described as occurring in rats fed on diets deficient in vitamin E. In testicular degeneration due to deficiency of this factor the organs are usually well below the normal weight(3) whereas in the condition under description their weight was, in general, greatly increased. This increase in weight was, however, due solely to the accumulation of dropsical fluid; without the effusion the weight of the organ, as judged from its microscopical appearances, would have been below normal. It is possible that the excessive accumulation of fluid bore some relation to the length of time the experiment lasted: 515 days.

This condition having been observed in the course of an experiment designed for another purpose, it is not possible to express a definite opinion as to its causation. It seems not unlikely, however, to be a late manifestation of vitamin E deficiency.

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PLATE LXIX.



FIG 1



FIG 2

EXPLANATION OF PLATE LXIX

- Fig 1 *Hydrops testis* in rat fed on the *cambu* diet for 515 days Weight of animal,  
152 grammes , weight of dropsical testes, 3.4 grammes  
,, 2 Testes of healthy, well-fed rat Weight of animal, 150 grammes , weight of  
testes, 1.5 grammes



EXPLANATION OF PLATE LXX

- Fig 3 Showing seminiferous tubules widely separated by serous effusion From a case of *Hydrops testis* in rat
- „ 4 Relatively normal seminiferous tubule from a case of *Hydrops testis* in rat
- „ 5 Seminiferous tubule from a case of *Hydrops testis* in rat , showing disruption of its compact structure, but otherwise relatively normal
- „ 6 Seminiferous tubule from a case of *Hydrops testis* in rat , showing active spermatogenesis



FIG 3



FIG 4



FIG 5



FIG 6



# EXPLANATION OF PLATE LXXI

- Fig 7 Seminiferous tubule from a case of *Hydrops testis* in rat, showing  
commencing degeneration of spermatids
- „ 8 Seminiferous tubule from a case of *Hydrops testis* in rat, showing early  
degenerative changes in all cellular elements
- „ 9 Degenerating seminiferous tubule from a case of *Hydrops testis* in rat
- „ 10 Complete degeneration of seminiferous tubule from a case of *Hydrops testis*  
in rat



# THE EXPERIMENTAL PRODUCTION OF STONE-IN-THE-BLADDER

## Part VII.

BY

COLONEL R. MCCARRISON, C.I.I., R.H.F. M.D., F.R.C.P., F.R.S.

*Director, Nutritional Research, I. R. F. A., Pasteur Institute, Coonoor, S. India*

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In a previous paper(2) it has been reported that the addition of whole milk to a stone-producing diet prevents the formation of urinary calculi in rats. The purpose of the present paper is to record an experiment, the results of which indicate that butter exercises a like protective action against this disease.

A group of 24 young rats (12 males and 12 females) were fed on the diet originally employed(1) for the production of stone in rats. This diet consists of oatmeal 53 parts, cornflour 25 parts, linseed meal 20 parts, sodium chloride 1 part, calcium phosphate 1 part and distilled water.

A second group of 24 young rats (12 males and 12 females) were at the same time, fed on a diet of similar composition but in which the linseed meal was replaced by butter. In all other respects the experimental conditions were the same in both groups.

The experiment, begun in the spring of 1929, lasted for 211 days.

Of the 24 animals fed on the diet *without butter* 7, or 29 per cent, were found at post-mortem examination to have urinary calculi (Table I). Of the 24 animals fed on the diet *with butter* none were found at post-mortem examination to have urinary calculi (Table II). Statistically speaking, the odds are, therefore, more than 100 to 1 in favour of this result being significant, and it may be concluded that the replacement of the linseed meal by butter afforded the rats protection against urinary calculus.

Details in regard to the 48 animals used in this experiment are given in the annexed Tables. From a study of them it will be seen that not only did the replacement of the linseed meal by butter afford the rats protection against urinary calculi but against the anæmia, the gastro-intestinal disease, the asthenia and the broncho-pneumonia from one or other of which certain animals, not receiving butter, suffered and died.

TABLE I

*Growing details regarding 24 rats fed on the oatmeal diet without butter*

Number of Animal	Sex	Original body-weight grams	Final body-weight grams	Cause of death	Days under experiment	Vesical calculus	Dilated ureters —— Cystitis	Diseased kidneys	Renal calculus
2387	M	34	42	Enteritis	42				
2388	F	32	47	Anæmia	64				
2389	M	35	200	Killed	211				
2390	F	35	126	Killed	211				
2391	M	36	63	Asthæmia (?)	85				
2392	F	37	62	Genito urinary disease	87	Gravel	Cystitis		
2393	M	32	63	Enteritis	73				
2394	F	32	42	Anæmia	66				
2395	M	31	136	Killed	211				
2396	F	33	79	Anæmia, prolapsed uterus, peritonitis	83	Gravel	Cystitis		

2397	M	31	82	Pneumonia	133			
2398	F	32	144	Killed	211			
2399	M	31	100	Killed	211			
2400	F	32	80	Killed	211	Stone		
2401	M	37	60	Broncho pneumonia	75			
2402	F	39	60	ditto	147			
2403	M	38	99	Undiscovered	123			
2404	F	38	65	Genito urinary disease	91	Stone	Cystitis	
2405	M	31	65	Gastro intestinal dystrophy	83			
2406	F	31	36	Inanition	62			
2407	M	31	88	Genito urinary disease	168	Stone	Cystitis	
2408	F	37	51	Anemia	65	Gravel	Cystitis	
2409	M	35	139	Killed	211			
2410	F	36	96	Genito urinary disease	161	Stone	Dilated ureters, cystitis	Pyonephrosis Gravel



TABLE II.

*Giving details regarding 24 rats fed on the oatmeal diet with butter*

Number of Animal	Sex	Original body weight grms	Final body-weight grms	Cause of death	Days under experiment	Vesical calculus	Dilated ureters — Cystitis	Diseased kidneys	Renal calculus
2411	M	33	113	Pneumonia	136				
2412	F	32	172	Killed	211				
2413	M	33	228	Killed	211				
2414	F	34	129	Killed	211				
2415	M	33	208	Killed	211				
2416	F	32	119	Killed	211				
2417	M	35	163	Killed	211				
2418	F	39	128	Killed	211				
2419	M	36	214	Killed	211				
2420	F	38	126	Killed	211				
2421	M	32	223	Killed	211				
2422	F	38	148	Killed	211				
2423	M	33	206	Killed	211				
2424	F	38	143	Killed	211				
2425	M	32	192	Killed	211				
2426	F	31	135	Killed	211				
2427	M	33	231	Killed	211				
2428	F	35	122	Killed	211				
2429	M	33	222	Killed	211				
2430	F	37	135	Undiscovered	171				
2431	M	32	169	Killed	211				
2432	F	32	142	Killed	211				
2433	M	31	116	Killed	211				
2434	F	35	102	Killed	211				

## CONCLUSION

A liberal supply of butter in a diet, composed of oatmeal, cornflour, sodium chloride and calcium phosphate prevents the formation of urinary calculi in rats

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# A STUDY OF THE INFLUENCE OF RELATIVE HUMIDITY ON THE LIFE AND INFECTIBILITY OF THE MOSQUITO

BY

BRUCE MAYNE

(*Malariaologist Malania Survey of India*)

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## INTRODUCTION

CONSIDERING that the mosquito is in a measure an amphibious insect it appears surprising what little regard has been paid to its moisture requirements relative to its behaviour towards man. Only recently has there been a definite protest against the attitude of regarding the temperature relationships of malaria transmission as all sufficient and significant. The excellent work of Jansco (1904) on the temperature requirements of plasmodial development in the mosquito has always been considered as ample criteria, a working model, for meteorological reaction. This work has been quoted repeatedly without regard to other meteorological factors functioning in successful conveyance of malaria. An appreciation of the habits and reactions of the insect carrier becomes increasingly desirable. This has not been lost sight of by the 'control worker' who has taken substantial due from the insect's biology availing himself of a knowledge of vulnerable points in the life-history and habits of the mosquito.

The early work of Bacot (1916) and Pierce (1916) probably laid the foundation for the newer researches in a rational interpretation of insect behaviour toward its environment. This was applied specifically in medical entomology by Gill (1921) and Buxton (1923), so that the way is unimpeded for a correct evaluation of insect reactions in their adaptations to tropical medicine and the human host.

Other authors who have recognized a correlative factor in humidity are mentioned as follows —

Hodgson, E. C., and Barraud, P. J. (1919) state that the mosquito in spite of being cold-blooded does not live at the temperature of the air, but at or about the temperature of the wet bulb thermometer in the hygrometer. It is described by them as a moist insect protected by a thick impervious cuticle traversed by innumerable thin-walled tracheæ. These are in direct communication with every

cell and also with the outside air through the spiracles, therefore, owing to the evaporation taking place through these air tubes the temperature of the mosquito must be at or near that of the wet bulb thermometer placed in an analogous situation

Buxton (1923), alluding to the seasonal prevalence of an insect, remarks that the two factors temperature and humidity, must be considered together, they are observed to interact on one another, and cannot be discussed independently. In fact, a certain temperature is only optimum at or about a certain relative humidity, and under other conditions of humidity that temperature may be far from favourable

Neeheles (1925) referring to the influence of humidity on the habits of *Anopheles maculipennis* observes that this species in its habit of flying only at certain hours of the day and its choice of hibernating places is largely dependent on the humidity and temperature of the environment. He explains that, placed in high temperatures, mosquitoes lower their body temperature by evaporating water, and therefore an air humidity above or below certain limits creates unphysiological conditions

Chapman (1926) writes that the dry bulb temperature of the air is not a reliable index of the insect's environment. When temperature and humidity are combined, the zones of *equal effect* are much more reliable. He states that the effect of extremes of temperature is modified by the moisture conditions of the organism

One can draw an analogy between the blood-sucking mosquito which when blood engorged, has an internal humidity of 100 per cent and is not influenced so readily by changes in external relative humidity, and an insect which feeds on the sap of plants. The latter, while the host plant furnishes an abundance of dilute sap, may be relatively independent of the direct effect of the humidity of the air. Desiccation is probably more severely felt by a starved or partly engorged mosquito, judging from experiences in numerous feeding tests

Wenyon (1926) considers that there is no evidence that the factor of the effect of humidity of the atmosphere plays any part on the active development of plasmodia in mosquitoes. Provided there is sufficient moisture in the air to enable the mosquito to live, the malaria parasite will develop normally. He regards temperature as a much more important factor than humidity, and refers to Gill (1921) who, however, has pointed out that the spread of malaria may be affected by the lack of humidity, because the mosquitoes which ingest parasites may not live long enough for sporozoites to appear in the salivary glands

As pointed out by Beattie (1928) there are definite boundaries of temperature and humidity which limit the metabolism and activity of insects. An optimum range is accepted as the degree of atmospheric humidity most favourable to the maximum rate of metabolism for each insect. The 'water optimum' of Headlee quoted by Beattie indicates the relationship of the insect to humidity independent of the factor of the body fluid being above this optimum or below

The impression is general amongst authors that if the conditions of relative humidity are favourable for the maintenance of life of the insect host the requirements of humidity are satisfied as regards malaria infectivity. Neumann (1909) modifies this view finding in his experiments with *Culex pipiens* and *Stegomyia fasciata* that if the humidity is lowered to about 10 per cent the development of the cysts is prolonged for at least two days. Unfortunately the temperature is not stated and he writes: 'So it seems that a dry atmosphere is relatively unfavourable to the infection of mosquitoes.'

Gill (1928) states finally that an important conclusion derived from an experimental study was that the degree of atmospheric humidity provided it was favourable to the life of the insect host did not appear to exercise any influence either on the number or the rate of development of malaria oocysts in the midgut of the mosquito. Humidity then exercises no direct effect upon the malaria parasite during its extracorporeal phase.

When the atmospheric humidity falls below the 'critical' figure (a constant relative humidity of 48 percentage at a temperature of 27°C in the case of *C. fatigans*) Gill found that infected mosquitoes do not survive long enough to enable them to transmit infection. It was shown that whilst infected and uninfected mosquitoes reacted in a similar manner to atmospheric states the temperature and humidity conditions compatible with the transmission of malaria were confined to a more restricted range than those favourable to the bionomics of the insect carrier.

The foregoing remarks of Gill epitomize essentially the pertinent references serving the purpose of the present paper. The present investigation was directly stimulated by the work of Gill in India whose results have been substantially confirmed and extended. His experimental data (Gill, 1921b) are summarized in Table I which includes two series of figures grouped as shown for the sake of comparison of results.

#### PROCEDURE FOLLOWED IN THE PRESENT EXPERIMENTS

The method advocated by Gill of keeping experimental mosquitoes inside glass bottles supplied with raucins and green twigs, in a 'mosquito hotel,' was not employed. Here only one end of the container was covered with cotton net, hence the desiccated air could not freely circulate. I preferred the use of small bulbous-shaped lantern globes with both open ends covered by coarse meshed cotton netting held with elastic bands (Plate LXXII, fig 1). Each of these confined three specimens of mosquitoes and as many as 200 globes placed side by side with both ends unobstructed could be accommodated in the large sized low temperature type incubators. The control specimens, maintained under optimum laboratory conditions, were kept in similar glass globes on their sides (Plate LXXII, fig 2). The wet and dry bulb thermometers were placed in the midst of the containers and in actual contact with the glass sides of the cages. When more than one tray was required in the incubator special drawers made of coarse meshed wire netting were employed

#### EXPLANATION OF PLATE LXXII

- Fig. 1 Type of glass cage ( $\times 1\frac{1}{2}$ ) with raisin held between layers of netted cloth used for exposure of mosquitoes to desiccated atmosphere
- „ 2. *A*—Lantern globes laid on their sides for experiments with mosquitoes where maximum desiccation was required  
*B*—Similar glass containers provided with moist pads in moist lint lined trays used when the maximum of humidity is desirable
- „ 3. Interior of incubator with two wire trays covered with glass cages arranged for exposure to fumes of sulphuric acid in glass desiccator
- .

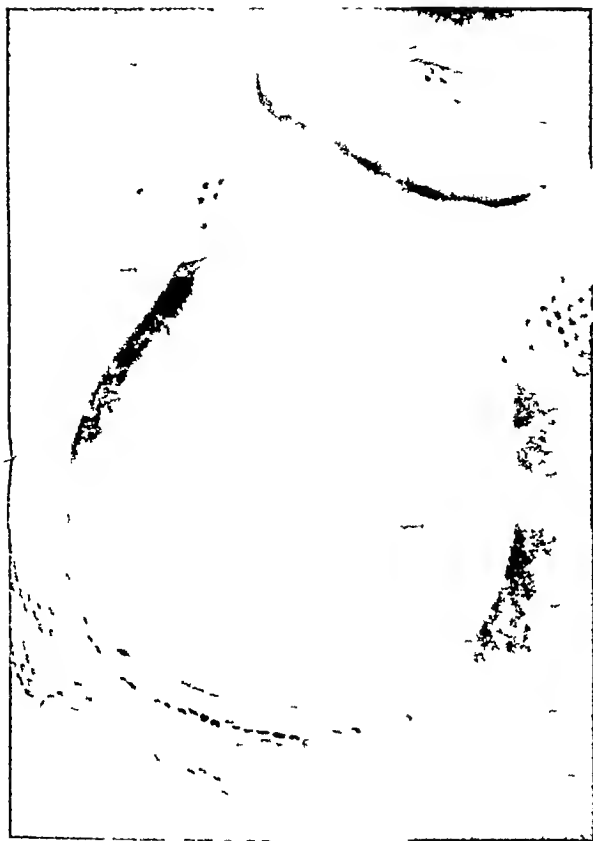


FIG 1



FIG 3



A

FIG 2

B



to support the glass cages, a wet and dry bulb thermometer being placed in each drawer (Plate LXXII, fig 3) The readings were taken twice daily, approximately at 7 a.m. and 8 p.m.

Owing to the tendency of the caged birds to attack the mosquitoes within reach and the consequent wholesale destruction of the insects it was found necessary to remove them as soon as practicable after engorgement. At first masks for the eyes of the bird and gauze bandages to render the sparrow helpless were used, but finally a more practicable method was employed. The mosquitoes kept for one to two days after emergence in large netted lintern chimneys, were placed in the feeding cage at a convenient time in the evening. The bird or birds in small cages made of wire and wicker were placed at the other end of the long feeding cage. After dark, when the birds were in repose, an arm was thrust through a sleeve in the cage and the netted tops of the chimneys were removed with their elastic bands thus liberating the mosquitoes. Several wet cloths were placed around the outside cage and the whole covered with a heavy dark material. The latter expedient was for the purpose of protecting the engorged mosquitoes within reach of the awakening birds in the early morning hours. The cloth coverings were taken off in the morning, the bird cages cautiously removed and the engorged mosquitoes collected in individual glass tubes through a sleeve in the feeding cage and then transferred into suitable globes.

Chemically pure sulphuric acid was used at first in conjunction with calcium chloride then without the latter for reducing the moisture content of the incubating chambers. It was found by actual test that it was safe to employ as much as six pounds of sulphuric acid in an open vessel in an incubator of cubic content less than three feet. This confirms Gill's experience in mosquito desiccation tests and follows the accepted procedure of other biologists. Stevens (1916), in devising a method for studying the humidity relations of fungi in culture, states — 'Other hygroscopic substances might be used to maintain a constant humidity, but sulphuric acid is convenient to use and so slightly volatile that it apparently does not affect the growth of the fungus.'

At the beginning of the humidity tests mosquitoes were placed in netted glass lamp chimneys on wooden trays lined with moistened lint. This was discontinued after demonstrating that the moisture thus unintentionally introduced when the incubator was filled with trays made a difference of 3° to 5° in the wet bulb thermometer, causing an actual rise of 12 percentage to 20 percentage in relative humidity. Subsequently the incubators were provided with wide mesh wire trays in place of the wooden ones and the glass cages were placed on their sides with both netted ends exposed to the action of the sulphuric acid.

It was found in practice that mosquitoes exposed to such conditions of maximum desiccation were unable to survive for more than 36 hours. The method of Gill was therefore adopted of furnishing the essential moisture by giving the caged insects a 'drink' of water from a cotton gauze sponge held against the netting from the outside. Three to five minutes were therefore lost in the



daily record of each lot of specimens exposed to the humidity tests on account of the necessity of removing the cages for the purpose indicated

# TABULATED RESULTS OF EXPERIMENTS

Table I represents the experimental data published by Colonel C A Gill on humidity affecting *C fatigans* and *Pl praeor*

## TABLE I

### Summary of Gill's experimental data

Percentage range, mean relative humidity	Mean temperature range °F	Number of specimens used	Number surviving	Number infected	Number of days exposed
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### Series A Infected *C fatigans*

100	77 1—83 6	15	13	13	5—8
83—89	78 8—83 9	82	65	49	5—10
60—61 3	80 6—82 4	32	23	10	7—10
48—48 3	78 8—81 5	20	9	7	10
46 4	79 8	11	2	2	5
42 0	80 6—81 7	21	1	1	5
40 4	81 9	8	0	0	5

### Series B Non-infected *C fatigans*

87 0	81 5	20	16		10
86 6	85 1	80	35		5
65 0	85 1	80	21		5
48 0	77 4	20	0		3
45 4	79 4	20	2		5
40 6	81 0	80	0		5

### Summary of Table I

The experimental data of Colonel Gill's article are arranged for convenience by me in 2 series of tables depending on the factor of the presence or absence of malaria parasites in the mosquitoes

In Series B at relative humidities under 48 percentage only 2 specimens of *C fatigans* survived as long as 5 days (long enough to insure *Proteosoma* infection) Whilst of 180 specimens exposed for 5 to 10 days to relative humidities of 65 percentage and above, 72 mosquitoes, or 40 per cent survived

In Series A, 40 specimens of *C fatigans* were kept for 5 days after biting malaria-infected birds Three of these survived at relative humidities under 48 percentage and proved to harbour malaria parasites At 48 percentage of relative humidity, 7 out of 9 survivors kept for 10 days showed plasmodial infection Above 60 percentage of relative humidity 101 mosquitoes lived for 5 to 10 days, of which more than 71 per cent (72 specimens) proved infected

I have made an effort to duplicate the essential factors reported by Gill in his experiments. Common English sparrows captured locally were employed to infect a given number of *Culex fatigans* bred from larva. The parasite employed was provisionally identified as *Plasmodium praecox* (Grassi and Feletti, 1904).

TABLE II

*Reaction of C. fatigans to various humidities at temperatures under 80°F*

Range of humidities	Range of wet bulb thermometer	Range of dry bulb thermometer	Number of days developed	Number of mosquitoes applied	Gametes per field in bird used	Mosquitoes infected and percentage	Extent of parasitism
36—39	50	62—63	3—5	92	1—8	0	Absent
41—42	53—59	66—73	12—16	103	1—22	$\frac{4}{38}$	Immature pigmented oocysts only
44—46	55—59.5	67—72	10—13	114	1—20	$\frac{77}{67.5}$	66 with sporozoites and oocysts, 11 oocysts various stages
47—48	50—51	60—61	14—19	121	1—20	$\frac{2}{16}$	Half grown pigmented oocysts only
49—50	62—65	74—75	14—21	29	1—135	$\frac{26}{89.6}$	3 with oocysts only, 23 with sporozoites and oocysts
51—55	52—55	60—65	19—23	76	1—37	$\frac{1}{13}$	Young pigmented oocysts only
58—64	55—59	63—66	15—24	68	1—5	$\frac{0}{0}$	Absent
60—62	68—70	78—79	12—14	101	1—200	$\frac{74}{73.2}$	All with oocysts and sporozoites lightly infected
70—75	57—64	61—69.5	7—12	98	1—19	$\frac{65}{66.3}$	Oocysts and sporozoites moderate numbers
84—85	58—61	60—64	11—15	42	1—48	$\frac{2}{4.7}$	1—oocysts and sporozoites, 1—pigmented oocysts only
96—100	76—79	77.5—79	10—12	31	1—140	$\frac{30}{96.8}$	Oocysts and sporozoites in great numbers

### Summary of Table II

At temperatures under 67°F and at varying humidities infection resulted in only 3 examples of *C. fatigans* out of a total of 399. These were observed up to 24 days.

At relative humidities from 36 percentage to 42 percentage only 4 specimens survived to the stage of infection out of 195 mosquitoes fed on heavily infected birds. These infected mosquitoes were observed to harbour a few immature pigmented forms.

At temperatures up to 72°F and relative humidities of 44 to 46 percentage the minimum effective range was recorded. Out of 114 specimens fed on a heavily infected sparrow 77 specimens, 67 per cent, became infected, the majority of them with sporozoites.

Infection resulted at relative humidities above 48 percentage providing the dry bulb temperature registered nearly 70°F and above. Of this batch 195 specimens out of 259 fed (75 per cent) became infected.

In like manner I have collected the results of similar tests conducted at temperatures above 80°F and placed them in the following table —

TABLE III

*Reaction of C. fatigans to various humidities at temperatures above 80°F*

Humidity range	Wet bulb thermometer range	Dry bulb thermometer range	Number of days developed	Number of mosquitoes applied	Gametes per field, bird's bloods	Mosquitoes infected	Per cent infected	Extent of parasitism
40—42	67—77	80—87	1—4	128	1—15	0	0	Absent
42—43	65—77	80—96	2—3	112	1—13	2	1.7	A few pigmented oocysts only
44—46	67—76.5	81—93	12—16	223	1—185	91	40.8	23 with sporozoites and oocysts, 68 with oocysts only
47—48	69—70.5	83—85	13—20	94	1—19	89	94.6	83 with sporozoites and oocysts, 6 with oocysts only
49—53	72—74	85—87	5—14	120	1—72	101	84.1	82 with sporozoites, 19 oocysts only
55—58	73—81	85—94	15—23	212	1—8	94	44.3	All with sporozoites, many with oocysts, various stages
60—68	70—74	80—82	7—12	74	1—17	69	93.2	12 with oocysts and sporozoites, 57 with sporozoites only
70—78	73—84	80—90	10—32	189	1—12	180	95.2	3 with undischarged oocysts, remainder, sporozoites varying from few to swarming numbers
80—88	80—82	85—86	12—18	42	1—18	38	90.4	Numerous ruptured oocysts, sporozoites in great numbers

## Summary of Table III

Mosquitoes fed on moderately to heavily infected specimens kept for a period up to 32 days at temperatures of 80°F to 96°F were dissected with the following results —

Below 12 percentage relative humidity none became infected, although the 128 specimens were applied to a heavily infected bird

One hundred and twelve specimens kept at 42 percentage relative humidity survived 3 days only and resulted in only 2 infections with undeveloped oocysts

Relative humidity of from 11 to 16 percentage was observed to prove the minimum effective range under the conditions noted. Of this series 91 specimens surviving a period of 12 to 16 days out of a batch of 223 specimens (40 per cent) became infected

Above 16 percentage relative humidity a total of 731 specimens were applied. Fully 78 per cent of these a total of 571 mosquitoes survived to the stage of infection

The optimum requirements for infection appeared to be above 60 percentage relative humidity and a dry bulb temperature of 80°F to 90°F. Under these conditions a total of 305 mosquitoes surviving during a month's time after biting moderately parasitized sparrows resulted in an infection rate of more than 94 per cent

The same procedure and apparatus detailed in the foregoing experiments were employed with species of *Anopheles* collected during the months of November and December 1928. The following table summarizes data of tests in humidities varying as recorded at temperatures under 67°F

TABLE IV

*Anopheles* affected by humidities at temperatures under 67°F

Species	Humidity range	NOVEMBER-DECEMBER 1928			
		Wet bulb thermo meter range	Dry bulb thermo meter range	Number of mosquitoes exposed	Number of days surviving
<i>culicifacies</i>	38—39	48—50	59.5—62	22	1—3
	41—50	48.5—53	60—62	38	2—4
	51—55	52—55	61—64	120	3—4
	57—62	55—58	63—65	28	10—21
<i>subpictus</i>	38—39	48—50	59.5—62	210	1—2
	41—50	48.5—53	60—62	218	1—2
	51—55	52—53	61—64	421	5—8
	57—62	55—58	63—65	119	12—24
<i>fuliginosus</i>	66.5—84	59—61	64—66.5	130	10—33
	38—39	48—50	59.5—62	221	10—17
	41—50	48.5—53	60—62	39	12—24
	51—55	52—55	61—64	114	9—30
	57—62	55—58	63—65	97	8—31

## SPECIES ADAPTATION TO RELATIVE HUMIDITY

With the limited knowledge at one's disposal there is suggested a species adaptation to meteorological requirements. This is obtained from an experimental study of the minimum effective relative humidity range. And in a general way observations in Nature verify the experimental evidence, applicable only for the common Indian species so far studied. The range of minimum relative humidity allowing for an infective period at an optimum temperature for functioning, may be stated as follows —

<i>Percentage of relative humidity</i>	<i>Species of mosquito</i>
38—40	<i>Anopheles fuliginosus</i>
43—45	<i>Culex fatigans</i>
55—58	<i>A. subpictus</i>
	<i>A. stephensi</i>
57—62	<i>A. culicifacies</i>

Probably the most definite in behaviour relative to meteorological adaptation are *Anopheles fuliginosus* and *A. subpictus*. The former affords the better example. *A. fuliginosus* (in north central India at least) survives the lowest temperatures and also resists the greatest desiccation of the early hot season. In numerous instances *A. fuliginosus* was observed to be exceptionally abundant and it was the only species collected during the middle of March (1929) when relative humidities of 16 to 21 percentage were recorded.

## DOES THE MALARIA SPOROZOITE REMAIN VIABLE IN THE ÆSTIVATING MOSQUITO ?

To determine if drought following normal high relative humidity will affect development of sporozoites, 151 specimens of *C. fatigans* after biting a heavily infected sparrow were placed in suitable small cages and exposed to a mean relative humidity of about 80 percentage (wet bulb 80°, dry bulb 85°) for a period of 10 days, then with a loss of 4 mosquitoes 47 specimens were left undisturbed, and the remainder placed in a desiccating incubator at about the same temperatures with a relative humidity of 44 to 50 percentage for a period of a maximum of 24 days. That there is no influence on the life of either host or parasite is indicated in the accompanying table (Table VII). Conversely, several tests indicated that mosquitoes enfeebled by a long period of debilitating low humidity were revived and behaved normally when subsequently maintained at a higher relative humidity. This was observed when *C. fatigans* and *A. culicifacies* subjected to relative humidities of 50 percentage and 56 percentage respectively, were removed and exposed at about the same temperatures for an appreciable time to mean relative humidities of 76 percentage and above.

TABLE VII

*Comparison of the effects of drought and that of high humidities on the infection of C. fatigans*

SPECIMENS EXPOSED TO 44 TO 50 PER CENT HUMIDITY		Day of experiment	CONTROL SPECIMENS AT OR NEAR 80 PER CENT HUMIDITY	
Number of mosquitoes dissected	Number infected		Number of mosquitoes dissected	Number infected
3	2	12	1	1
8	6	16		
		17	3	2
		18	2	2
6	1	19		
1	3	20	1	0
3	2	22		
3	'	23	3	2
		24	2	1
11	9	25	4	3
5	1	26	2	2
		27	4	3
6	1	29	2	2
13	11	30	3	2
9	7	31	2	1
12	11	32	3	2
3	2	33	2	2
14	10	34	13	9

*Summary of Table VII*

In exposing 100 mosquitoes to a mean relative humidity of 44 to 50 percentage at mean temperatures of 84 to 87°F, 78 per cent became infected up to 34 days. At the end of this time 14 per cent of them were alive. These were killed for purpose of dissection and examination.

A control series of 47 specimens which had up to 12 days previously been kept at the same humidity as the other lot were subjected to a continuous high relative humidity of above 80 percentage. Of this lot 72 per cent became infected. At the end of 34 days 27 per cent remained to be disposed of. These were killed and examined for evidence of infection.

The infection rate in these two series proved that exposure to a desiccating atmosphere following a period of normal high humidity did not interfere with infection, as demonstrated by an examination of dissected mosquitoes

#### HUMIDITY REACTIONS AND BITING STIMULI

Of crucial import is the stimulus affecting biting of the host, without which the condition of parasitism in the mosquito is after all secondary. The relations of temperature and humidity associated with the primary consideration of inducing blood ingestion should be well defined. Referring to the literature, one's impressions on this score appear hazy, and when subjected to critical analysis may be regarded as inaccurate and stereotyped. As the result of the intensive study of the insects' behaviour with particular stress on humidity reactions and biting stimuli, one is inclined to become more circumspect in the biological interpretation of a simple phenomenon. Is not one justified in assuming that in the case of most mosquitoes the stimulus to biting is provided by an environment of increased humidity in an optimum temperature rather than by the absence of light or other factors to which this phenomenon has been ascribed?

I had planned to conduct biting tests with species of *Anopheles* and a human host, using a specially constructed desiccator at various relative humidities. This project was dismissed for lack of funds, so one had resort to experiments with birds and *C. fatigans*. The following table (Table VIII) summarizes the results of ten experiments which were conducted with eight distinct batches of bred mosquitoes employed 48 hours or more following emergence.

TABLE VIII

*Influence of relative humidity on biting stimulus of C. fatigans*

Date	Wet bulb reading	Dry bulb reading	Relative humidity	Number of mosquitoes liberated	Number of mosquitoes blood engorged	Batch of mosquitoes used
8th July, 1929	80	95.5	52	325	0	A
6th „ „	74.5	90	49	298	0	A
7th „ „	76	91.5	50	300	0	B
8th „ „	80	95.5	52	258	3	C
12th „ „	80	92.5	58.5	286	12	C
14th „ „	86	88	97	120	73	D
16th „ „	87	89	93	216	122	E
19th „ „	81	88	74	97	42	F
20th „ „	81	86	81	47	36	G
22nd „ „	80	84	84	42	32	H

*Summary of Table VIII*

The results of these biting tests satisfy in a measure the query as to whether mosquitoes (*C. fatigans*) can be induced to bite in an atmosphere of relative humidity in which they are unable to survive desiccation following a blood meal. The results indicate that in the presence of a relatively high temperature biting does not occur at relative humidities under 52 percentage.

It must be pointed out that in actual experimental procedure a difficulty should be anticipated in regard to accurately gauging the influence of the host's body on the contiguous atmospheric humidity. This may be indicated in a rough way by first measuring with a sling psychrometer the relative humidity in a room, after which an animal in this instance a buffalo, is introduced. After a few minutes in the presence of the animal the sling psychrometer is used for a second reading. The instrument is swung about the animal's body as well as in the room generally. I have found in this crude test an actual increase in relative humidity of 4 percentage. Doubtless an increased number of animals would materially affect the relative humidity of a confined space.

Another way (perhaps less crude) of demonstrating the effect of one's body on the degree of relative humidity is by placing one's arm close to the sensitive member of a hair hygrometer and noting the rise on the chart of the revolving gauge. I have observed during the dry season an increase of 8 percentage of relative humidity in a short exposure of this sort.

## TEMPERATURE REQUIRED FOR BITING AND THAT OF GAMETOGENESIS

In a consideration of the active functioning of infectivity it must be stressed that relative humidity *per se* cannot be effective without constant reference to the dry bulb thermometer. A high relative humidity in the presence of low temperature may be conducive to stimulation of biting and yet be ineffective. This has been indicated in the foregoing experiments. Gill (1921b) stated that a fall in mean temperature in spite of a concomitant rise in relative humidity may cause the cessation of infection.

I have personally observed instances of biting of anophelines at temperatures as low as 58°F in the presence of a high humidity, and Gill (1914) reports observations in which *A. subpictus* and *A. fuliginosus* were induced to bite at temperatures as low as 54°F.

It may be suggested that observations of this type are of real value and probably more significant than many laboratory tests conducted under artificial conditions.

## THE INFLUENCE OF HEAT ON THE LIFE OF MOSQUITO AND ON ITS PARASITES

It was observed in desiccation experiments, that providing the humidity was favourable, mosquitoes could survive 15 to 23 days at temperatures as high as 94°F and in the presence of unfavourable relative humidity (42 to 43 percentage) for 2 to 3



days at temperatures as high as 96°F. In many instances the mosquitoes developed heavy infections and no deleterious effect on the malaria organism was noted.

The thermal death point of the mosquito (*C. fatigans*) was determined with the following results:

At a relative humidity of 55 to 57 percentage and at temperatures of 40°C to 42°C (104°F to 107.6°F) the mosquitoes lived for one to three hours.

At 43°C (109.4°F) maintained for 30 minutes the mosquitoes were disabled but recovered within twenty minutes, subsequently dying after one hour.

At 48°C (118.4°F) the mosquitoes lived less than one minute. The thermal death point may be regarded as about 45°C with a mean relative humidity of 56 percentage.

Living sporozoites were recovered on dissection at the following temperatures 42°C and 48°C. In the former the parasites appeared quite normal in appearance and motility, at the higher temperature the glands examined proved heavily infected, the sporozoites being packed in heavy agglutinating masses. The motility though definite was not typical, and from the hypertrophied appearance of some of the organisms an abnormal effect was demonstrated.

#### DISCUSSION

The question of the effect of relative humidity on the dissemination of malaria resolves itself into the question of the influence of temperature and humidity on both the insect carrier and the parasites it harbours. We are concerned primarily with the minimum effective range on the life of the insect. It has been demonstrated that if the relative humidity inhibits the development of the malaria organism in a hospitable mosquito one must satisfy oneself that the temperature is effective for gametogenesis. After this factor is determined one may be secure in stating that provided that the mosquito survives, the development of the plasmodia is not interfered with whatever may be the degree of desiccation.

This principle cannot be applied to parasitology generally. In one instance Cleveland (1923-1924) quoted by Brues (1927) has found that termites survive desiccation when their intestinal protozoan parasites are destroyed. It was determined that the thermal death point of the termite host was as high as 48°C while its parasite succumbed to an exposure of 36°C temperature for a period of 24 hours.

The important relationship of relative humidity to the biting stimulus in mosquitoes is definitely impressed in the results shown. The minimum relative humidity which induces biting in the mosquito is sufficient to permit of gametogenesis and subsequent development of the plasmodia ingested with the blood by the insect host.

On the other hand the minimum temperature at which culicines will bite birds or that at which anophelines may be induced to bite man is substantially lower than that required for gametogenesis and further development of the malaria parasite imbibed by the mosquito.

In conclusion one may impress the need for expression of relation of temperature and humidity to insect life and the parasite it harbours with a more intimate degree of accuracy. One is inclined to endorse the views of Buxton (1928) who justly complains that the great mass of data relating to climate in all parts of the world has been accumulated by professional meteorologists whose objective is the study of the dynamics of the atmosphere. This data is not available for the biologist who wants to know about climate as it affects man, animal or plant. Shade temperature, taken in a double louvered cage (Stevenson's screen), and rain measured in a gauge in the middle of an open space, are of slight interest to the medical biologist. It is essential that we study the conditions in the swamp, the forest and jungle, the animal quarters and human habitations. We must, moreover, study a great range of climatic factors such as solar radiant heat, the seasonal incidence of ultra violet radiation, the intensity of light, the prevalence of different types of rainfall. And regarding the latter it is clear that an inch of rain falling in half an hour is biologically entirely different from the same quantity of rain spread over 24 hours. This he asserts is the biologist's vision of what should free us of a grave deficiency in knowledge in the past.

Finally one can discern that the important question of species sanitation in malaria control must include as of equal sanitary significance the meteorological conditions of *species adaptation*.

#### SUMMARY

In experiments with *Culex fatigans* and organisms of bird malaria the minimum effective range of humidities permitting of both life of the mosquito and viability of the unhibed malaria organism was observed to be 44 to 46 percentage in both series of mosquitoes maintained at temperatures up to 72°F and at temperatures above 80°F.

At relative humidities under 42 percentage and dry bulb temperatures of 73°F or under, out of a total of 195 mosquitoes applied only 4 specimens were induced to bite and these became infected. At temperatures above 80°F at similar relative humidities none of 128 applied survived more than 4 days nor became infected. At equal temperature and 43 percentage humidity two specimens of mosquitoes survived 3 days, showing incipient parasite development.

In similar tests with anophelines at temperatures under 67°F and relative humidities from 38 to 55 percentage *A. culicifacies* and *A. subpictus* failed to survive one week, while more than 200 specimens of *A. fuliginosus* survived fully 17 days at a minimum relative humidity of 38 percentage. At temperatures above 80°F these three species failed to resist a desiccating atmosphere of 40 to 42 percentage for more than 24 hours.

At relative humidities ranging from 55 percentage and above at temperatures above 80°F and at relative humidities above 57 percentage and at temperatures below 67°F all three species survived from 1 to 4 weeks.

The experiments on the effect of relative humidity on the development of the malaria parasites in *Anopheles subpictus* and *A. stephensi* resulted in survival and infection of 2 specimens of *A. subpictus* in 1 to 3 days at 39 to 42 percentage relative humidity and 4 specimens during a period of 6 to 13 days at relative humidity of 54 to 59 percentage. In the latter test, development in the mosquitoes was carried to the sporozoite stage.

In the instance of six specimens of *A. stephensi* which succeeded in biting the patient, three of them during a course of 5 to 11 days at relative humidities of 80 to 88 per cent demonstrated various stages of Plasmodian infection. Only one showed a few gland sporozoites.

In these meteorological tests there was a definite result obtained indicating a species adaptation to relative humidity. This consisted of an effective range of relative humidity allowing for an infective period in the mosquito at an optimum temperature for functioning. The following range of percentage of minimum relative humidity was established: *A. fuliginosus* 38 to 40, *Culex fatigans* 43 to 45, *A. subpictus* and *A. stephensi* 55 to 58, and *A. culicifacies* 57 to 62.

It was determined, for an aestivating period involving a relative humidity of 44 to 50 percentage, that infected mosquitoes (*C. fatigans*) survived for periods up to more than 3 weeks (24 days). These had been maintained at normal high humidities for 10 days previously. Seventy-eight per cent of these retained their malaria parasites during the period of enforced drought, contrasted with 72 per cent of control specimen, which remained infected through 34 days of the experiment.

The relation of relative humidity to the stimulus of seeking blood in the mosquito was tested. The results of these biting tests indicate that biting does not occur at relative humidities under 52 percentage even in the presence of high dry bulb temperatures. This demonstrates that this species of mosquito (*C. fatigans*) cannot be induced to bite in an atmosphere of relative humidity in which it is unable to survive desiccation.

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AN INVESTIGATION TO DECIDE THE MOST SUITABLE  
DILUTIONS FOR THE PERFORMANCE OF THE  
ANTIMONY TEST FOR KALA-AZAR

BY

L EVERARD NAPIER, M R C S (Eng), L R C P (Lond),

AND

G N SEN, M D (Cal),

*Clinical Assistant to the Kala azar Ancillary Inquiry under the Indian Research  
Fund Association*

*(From the Kala azar Research Department, Calcutta School of Tropical  
Medicine and Hygiene)*

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LIEUT-COLONEL R N CHOPRA and his co-workers in the Pharmacological Department of the Calcutta School of Tropical Medicine observed that the addition of a 4 per cent solution of certain pentavalent compounds of antimony to the serum of a kala-azar patient caused complete coagulation of the serum. After this original observation Col Chopra and the senior writer each worked out a technique for its application to the diagnosis of kala-azar. The former suggested that a 4 per cent solution of antimony should be added to undiluted serum but the latter was of the opinion that the method was not sufficiently critical and suggested using a weaker solution of the antimony compound. Later Col Chopra, also finding that his original test was not critical enough, suggested diluting the serum 8 to 10 times with distilled water, by this means he found that positive results in cases other than kala-azar were eliminated. The senior writer (Napier, 1927) suggested that by combining these various methods a quantitative test might be devised, the results of which, when considered in conjunction with the clinical picture, might give the clinician considerable assistance in arriving at a diagnosis. Bose, Dastidar and Bagehi (1928), possibly following on this suggestion, carried out the test using undiluted and two dilutions of serum, 1 in 5 and 1 in 10, and 3 strengths of urea-stibamine, 4 per cent, 2 per cent, and 1 per cent. Unfortunately all their cases were either advanced kala-azar with a strongly positive aldehyde reaction, or controls consisting of patients suffering from some disease or injury which could

not be mistaken for kala-azar, fractured clavicle for example, so that the results of the nine combinations were in most cases either all positive or all negative. As the aldehyde test has always given entirely satisfactory results in the diagnosis of these long-standing cases, no advance was established by the introduction of a technique which is more troublesome.

Col Chopra's and the senior writer's choice of the strength of urca-stibamine was in each case more or less arbitrary, the former chose 4 per cent because it happened to be the strength which he was using when he made his original observation, and the latter chose 0.25 per cent as being the weakest concentration with which a heavy precipitate was obtained in a well-developed case of kala-azar, but in neither instance was the choice dependent on using a number of different dilutions in an extensive clinical trial. The present writers, therefore, decided that it would be worth while investigating this point and carried out tests with a number of different dilutions of the serum and varying strengths of urca-stibamine with the object of ascertaining if a particular combination would give a better result than the rest.

#### THE PATIENTS

For this purpose we decided that the test must be carried out in a number of cases in which a definite diagnosis of kala-azar had been made, either by finding the parasite, or by establishing its absence by means of a culture on N N N medium of spleen or liver puncture material. As our object was to find a test which was more specific than the aldehyde test, at first we took only cases in which the aldehyde test gave an inconclusive result, they were mostly cases in which the aldehyde test was doubtful, but in a few of them, although the aldehyde test was negative, there were strong clinical grounds for suspecting kala-azar. In nearly every case the diagnosis was made after the test was done. The serum of over 60 cases were tested. In a few the culture was contaminated, such cases were excluded from the series. Of the remainder the first 25 which showed leishmania and the first 25 which showed no leishmania were taken for comparison, and the results of these appear in Tables V and VI. As we felt that these results might mislead anybody who read the paper hurriedly into doubting the value of the antimony test altogether, we did two other series of tests, one of patients who were definitely diagnosed as kala-azar by the aldehyde test (subsequently confirmed by spleen puncture) and 25 in which the diagnosis of kala-azar did not arise. This latter group were all patients who were in hospital being treated for some other definite disease and, although a spleen or liver puncture was not done, it is highly improbable that any one of them was suffering from kala-azar.

#### THE TECHNIQUE

There are one or two difficulties that arise in connection with the technique of the antimony test described by Col Chopra, a difference of opinion is sure to exist between different observers as to what is a 'thick and flocculent' and a

'definite flocculent' precipitate, more especially when the dilute serum is used. Furthermore the narrow calibre of the tubes used causes slight variations in the method of adding the antimony to lead to different degrees of mixing and consequently to different readings. On more than one occasion completely opposite readings have been made by the same observer about different samples of the same serum. A small technical irregularity, such as a test tube not being scrupulously clean, has led to a precipitate forming in a serum which, tested in a clean tube was entirely negative. We were, therefore, very careful to use a uniform technique.

*The urea-stibamine* Brahmachari's brand of this preparation was used throughout. All the tubes were apparently from one batch as they were received in one box from Dr Brahmachari himself who very kindly supplied them free for experimental purposes. The antimony content was found to be 41 per cent. The urea-stibamine was dissolved in distilled water to make a 4 per cent solution. From this 2 per cent, 1 per cent and 0.5 per cent solutions were prepared.

*The serum* The blood was taken in the usual way and allowed to stand until it had clotted, the serum was then drawn off. We have observed before that serum when kept even in a cold incubator undergoes a change. On the addition of antimony compounds a more marked precipitate is formed with serum that has been kept for 24 hours than with the same serum immediately it has separated. We confirmed this observation by an experiment which is reported later in the paper. In order that the time factor should not have to be taken into consideration we kept all our sera 24 hours before performing the test. Four cubic centimetres of serum was necessary for each series of tests, 2 c.c. was placed in one tube and to the other tube containing 2 c.c. of serum an equal quantity of distilled water was added and the tube rotated between the palms to mix the contents, 2 c.c. was withdrawn this was again diluted with an equal quantity of distilled water, and the process repeated to obtain the third dilution. We thus had pure serum, half strength, quarter strength and 1-in-8 serum.

*The test* Seventeen test tubes, 6.5×1 centimetre (outside measurements), were stuck into a tray of plasticine, in 4 rows of 4 each, with one extra tube as a control. To the 4th tube of each row 0.5 c.c. of 1-in-8 serum was added by means of a measured pipette. Using the same pipette 0.5 c.c. of 1-in-4, 1-in-2, and undiluted serum were added to the 3rd, 2nd and 1st tubes in each row, respectively. Then the urea-stibamine solution was added, starting with a clean measured pipette, 0.5 c.c. of the 0.5 per cent solution was added to each tube of the 4th row, 1 per cent solution to each tube of the 3rd row, 2 per cent solution to the 2nd row, and 4 per cent to the 1st. Each tube was then shaken to complete the mixing and the readings taken immediately. The control tube contained 1-in-8 serum only.

*Reading of the results* The results could be classed in 3 groups, positive, doubtful and negative. If the mixture was so opaque that when it was held up



TAB

Showing slight variations in the reactions in the

Serial No	Aldehyde Reaction	4 PER CENT UREA STIBAMINE			2 PER CENT UREA STIBAMINE				
		Serum 100 per cent	Serum 50 per cent	Serum 25 per cent	Serum 12½ per cent	Serum 100 per cent	Serum 50 per cent	Serum 25 per cent	Serum 12½ per cent
(9)									
A	— ve	—	—	—	—	±	—	—	—
B	— ve	±	—	—	—	±	—	—	—
(33)									
A	+++	+++	+++	++(+)	+(+)	++	++(+)	++	+
B	+++	+++	+++	++	+	++	+++	+(+)	+
C	++	+++	+++	+++	+(+)	+++	++(+)	++	+
D	+++	+++	+++	++(+)	(+)	+++	++(+)	++	+
E	+++	+++	+++	++	+	++	++(+)	++(+)	+
(34)									
A	++	+++	+++	+(+)	±	++	++	(+)	±
B	++	+++	+++	++	—	++(+)	++	+	—
C	+	+	+++	++(+)	±	++(+)	++	+(+)	±
D	+	+++	++	+	±	++	+(+)	+	±
E	+	+++	++(+)	+(+)	±	++	++	+(+)	±

TAB

Showing variations in the reaction of the

Serial No	Test done	4 PER CENT UREA STIBAMINE				2 PER CENT UREA STIBAMINE			
		Serum 100 per cent	Serum 50 per cent	Serum 25 per cent	Serum 12½ per cent	Serum 100 per cent	Serum 50 per cent	Serum 25 per cent	Serum 12½ per cent
1	½ hour after blood taken	+++	+++	++	±	++(+)	++	+(+)	(+)
2	20 hours after blood taken	+++	+++	++(+)	(+)	++(+)	++(+)	++	(+)
3	96 hours after blood taken	+++	+++	++(+)	+	+(+)	++(+)	++(+)	+

## LE I

*serum of the same patient taken on different days*

1 PER CENT UREA STIBAMINE				$\frac{1}{2}$ PER CENT UREA STIBAMINE				Total number of positive readings
Serum 100 per cent	Serum 50 per cent	Serum 25 per cent	Serum 12 $\frac{1}{2}$ per cent	Serum 100 per cent	Serum 50 per cent	Serum 25 per cent	Serum 12 $\frac{1}{2}$ per cent	
±	—	—	—	—	—	—	—	0
±	±	—	—	±	±	—	—	0
(+)	— (+)	— (+)	+	±	+	+	(+)	13
+	± (+)	(+)		(+)	+	+	+	15
+	++	+	+	(+)	+	+	(+)	14
+	++	+	+	(+)	+	+	+	14
+	++	+	+	±	+	+	+	15
+(+)	— (+)	+	±	(+)	+	(+)	±	9
+(+)	++	—	±	(+)	+	(+)	±	10
++	++	+	±	(+)	+	+	±	11
+	+	+	±	(+)	+	(+)	±	10
+	+(+)	+	±	(+)	(+)	(+)	±	9

## LE II

*same serum tested after different intervals*

1 PER CENT UREA STIBAMINE				$\frac{1}{2}$ PER CENT UREA STIBAMINE				Total number of positive readings
Serum 100 per cent	Serum 50 per cent	Serum 25 per cent	Serum 12 $\frac{1}{2}$ per cent	Serum 100 per cent	Serum 50 per cent	Serum 25 per cent	Serum 12 $\frac{1}{2}$ per cent	
+	+(+)	+	(+)	±	+	+	(+)	11
+	++	+(+)	(+)	±	+	+	+	12
+	+(+)	+(+)	+	±	(+)	+	+	14



Serial No	1 PER CENT SOLUTION OF URIC ACID					ANTIMONY TEST COL CHOPRA'S TECHNIQUE				Number positive out of 16 tests
	Serum per cent	Serum 50 per cent	Serum 25 per cent	Serum 12.5 per cent	Abdo.lyde Reaction	DONE IN SENIOR WHITE'S LAB		DONE IN COL CHOPRA'S LAB		
						Undiluted	Diluted	Undiluted	Diluted	
1	+	++	++	++	+++	+++	+++	++	+++	16
2	+	(+)	(+)	—	+	+++	+++	++	+	6
3	+	(+)	(+)	(+)	+	+++	+++	++	++	8
4	+	+	+	(+)	+	+++	+++	++	++	11
5	+	+	+	(+)	++	+++	+++	++	+++	11
6	+	—	+	±	+++	+++	+++	++	++	11
7	+	+	+	+	+++	+++	+++	+	++	14
8	+	+(+)	+(+)	+(+)	+++	+++	+++	++	++	16
9	+	—	+	(+)	+++	+++	+++	++	++	13
10	+	+	(+)	±	++	+++	+++	++	++	10
11	+	+(+)	+(+)	+	+++			++	++	15
12	+	+	+	+	+	+++	+++	++	++	12
13	+	++	++	+(+)	+++	+++	+++	+	+	16
14	+	++	+(+)	(+)	+++	+++	+++	++	++	12
15	+	++	++	+	+++	+++	+++	++	±	15
16	+	++	++	+(+)	+++	+++	+++	++	++	16
17	+	+(+)	++	++	+++			++	++	16
18	+	+	+(+)	+(+)	+++	+++	+++	+++	++	15
19	+	+	+(+)	+(+)	+++	+++	+++	++	+++	15
20	+	++	++	+(+)	+++	+++	+++	++	++	16
21	+	++	++	+	+++	+++	+++			15
22	+	+(+)	+	(+)	+					12
23	+	++	++	+	+++	+++	+++	+	+	16
24	+	++	++(+)	+(+)	+++					16
25	+	++	++	+	+++					13
Number of positive results		23		16						
Column		N		P						

[illegible]

Serial No	1111 CRISTO STRIAND			ANIMONY TEST COL CHOPRA'S TECHNIQUE					Number positive out of 16 tests
	Serum 100 per cent	Serum 50 per cent	Serum 12.5 per cent	Aldehyde Reaction	Done in SENIOR WHITE'S LAB		Done in COL CHOPRA'S LAB		
					Un diluted	Diluted	Un diluted	Diluted	
1	+++	++	=	±	—	—	—	—	8
2	—	—	—	=	—	—	—	—	0
3	++	(-)	—	(-)	+	+	+++	±	1
4	(+)	(-)	±	(-)	—	—	+	—	1
5	+++	++ (-)	—	(-)	±	—	—	—	6
6	+++	—	—	±	+++	+++	—	—	5
7	+++	++ (-)	—	±	+++	+	—	—	6
8	+++	—	(-)	(-)	+++	+++	—	—	11
9	+++	++	=	(+)	+	+	++	+	11
10	+++	++ (+)	±	(+)	+++	+++	—	—	6
11	++ (+)	—	—	(+)	+	±	—	—	2
12	+++	++	=	(+)	+++	+++	—	—	9
13	(+)	=	—	±	—	—	—	—	1
14	+++	++	—	(+)	—	—	—	—	—
15	++ (+)	++	=	(+)	±	—	+	—	—
16	++	++ (+)	—	±	±	—	+	—	—
17	+++	++ (+)	±	±	±	—	+	—	—
18	+++	++ (-)	=	(+)	+++	+++	+++	—	—
19	+++	++ (+)	(+)	(+)	+	—	+	—	—
20	+++	++	=	(+)	—	—	—	—	—
21	+++	++ (+)	±	(+)	+++	+++	—	—	—
22	—	—	—	—	—	—	—	—	—
23	+++	++	±	(+)	+++	+++	+	—	—
24	(+)	+	—	±	±	+++	—	—	—
25	++	+	±	±	—	±	—	—	—
Number of positive results	20	21	0						
Column	A	B	P						

0.5 PER CLNT SOLUTION OF URIA-STIBAMIN				Aldehyde Reaction	ANTIMONY TEST COL CHOPRA'S TECHNIQUE				Number positive out of 16 tests
m nt	Serum 50 per cent	Serum 25 per cent	Serum 12 5 per cent		DONE IN SENIOR WRITER'S LAB		DONE IN COL CHOPRA'S LAB		
					Un diluted	Diluted	Un-diluted	Diluted	
	(+)	±	—	(+)	+++	++	+	±	6
)	+	(+)	—	±	+	+			7
	(+)	(+)	±	(+)	+	+	+	++	6
	(+)	(+)	±	(+)	—	—			5
	(+)	(+)	+	(+)	+++	+++			9
	±	±	—	±	+++	+			3
)	+	(+)	±	±	+++	+			7
	+	+	(+)	±	+++	+++			12
	+	(+)	—	(+)	+++	+++			8
	+	(+)	—	—	±	—			1
++	(+)	—	—	—	±	—			2
++(+)	+	—	—	—	±	—			2
	—	±	(+)	(+)	±	—			8
	(+)	—	—	(—)	+++	+			6
		—	—	(+)	+++	+++			8
		—	—	—					0
		—	—	(+)			++	±	6
		±	—	(+)			+	—	6
	+	±	—	—	—	—			0
		—	—	(+)	+++	+	+	±	5
		—	—	(+)	+++	+++	+	+	7
		±	—		±	—	+	—	3
		—	—		++	+++	+	+	11
		—	—		++	+	++	+	6
		—	—		+	±	+	—	4

In the same tables there are columns showing the results of the aldehyde reaction Col. Chopra's antimony tests with both diluted and undiluted serum done by himself or one of his assistants and also the results of the same two tests done according to Col. Chemist's technique by the senior writer. Lastly there is a column in which it is noted how many of the tests in the sixteen different combinations were positive. At the foot of each column there is a figure showing the number of positive results in that column.

*Comparison of results in cases of definite kala-azar and in cases of other diseases*  
Tables III and IV

Of the kala-azar cases 8 were positive throughout, 5 were positive in 15 out of 16, and a further 9 were positive in all 11 tubes or more. This leaves only three—these scored 10, 8, and 6 positives, respectively. The average of the whole group was 13.44 positives for each serum. In the non-kala-azar group one serum only obtained any positive results and this one only two. Then taking the different dilutions we see that in the first two columns (i.e. 4 per cent meta-stibamine with 100 per cent and 50 per cent serum) 26 results were positive of these 25 were cases of kala-azar and 24 were negative of which none were kala-azar—that is 49 (or 98 per cent) of results were correct. Whereas in the third dilution (i.e. 4 per cent meta-stibamine and 25 per cent serum) 25 results all cases of kala-azar were positive and the 25 non-kala-azar were negative—that is 50 (or 100 per cent) of the results were correct. Without discussing each dilution separately we can say that the results in columns C, L, I, and J gave 100 per cent correct readings and that in columns A, B, G, I, K, N, and O the readings were 88 per cent or more correct. This leaves 5 columns D, H, L, M, and P in which the results are not satisfactory, these include all the 1 in 8 dilution sera and one strong serum mixed with the most dilute of the meta-stibamine solutions. It is thus obvious that with this test there should be no difficulty in distinguishing between cases of these two groups. These results are shown in tabular form in Table VII.

*Comparison of the two groups clinically suggestive of kala-azar but in which the aldehyde reaction gave an indeterminate result* Tables V and VI

The position with reference to these two groups is quite different. Taking the number of positive results obtained in the case of each serum we find that the average number of positive results in the kala-azar group is 5.68, and the average in the other group is 5.52—that is to say there is practically no difference. Looking at it another way amongst the non-kashmania group only 3 cases gave more than 8 positive results whereas amongst the kala-azar group there are 6 cases giving 9 or more positive results. This difference is not however great enough to be significant and we must conclude that even by doing the whole sixteen tests in each case little help in making a diagnosis can be made amongst this class of patient.

One of the initial objects of this enquiry was to ascertain in which combination of dilutions of the serum and antimony solution the most specific results could be obtained. The Table below shows the total number of positive results in each combination of dilutions in each of the 4 groups of cases.



TABLE VII

DILUTIONS		Serum	NUMBER OF POSITIVE RESULTS IN		Correct diagnoses in 50 cases Tables III and IV	NUMBER OF POSITIVE RESULTS IN		Correct diagnoses in 50 cases Tables V and VI	Total correct diagnoses *
Urea stibamine			Table III Kala azar	Table IV Control		Table V Kala azar	Table VI Control		
A	4 per cent	100 per cent	25	1	49	20	23	22	71
B	"	50	25	1	49	21	19	27	76
C	"	25	25	0	50	6	5	26	76
D	"	12.5	12	0	37	0	0	25	62
E	2 per cent	100 per cent	25	0	50	20	22	23	73
F	"	50	25	0	50	18	17	26	76
G	"	25	24	0	49	7	5	27	76
H	"	12.5	15	0	40	0	0	25	65
I	1 per cent	100 per cent	23	0	18	12	14	23	71
J	"	50	25	0	50	17	17	25	75
K	"	25	24	0	49	7	4	28	77
L	"	12.5	15	0	40	0	0	25	65
M	0.5 per cent	100 per cent	13	0	38	3	3	25	63
N	"	50	23	0	48	6	6	25	73
O	"	25	22	0	47	4	2	27	74
P	"	12.5	16	0	41	0	0	25	66

\* Either positive or negative in 100 cases

There are in this table also columns showing the number of correct diagnoses with each combination. The results in the Tables V and VI show marked parallelism, and with no combination of dilutions is the correct diagnosis made on more than 28 occasions out of 50. As by tossing a coin very similar results would be obtained it cannot be said that the test with any of these combination of dilutions is likely to be of much value in this particular class of patient. Judging from the results in all four groups it is apparent that, although there is very little difference between the results in most of the various combinations the best are obtained with the 25 per cent and 50 per cent serum when these are added to 4 per cent, 2 per cent or 1 per cent urea-stibamine.

*Comparison of results obtained by our method and by Col Chopra's technique*

We were somewhat disappointed with these results. We had hoped to find that in one or more of the combinations more specific results would be obtained. It occurred to us that it might have been our method which was unsatisfactory and that Chopra's technique, though in our opinion very liable to be varied when applied by different observers, might bring into play some other specific physical factor. We, therefore, looked up the results of this test done by his method in the case notes of each patient. In some cases the test had been done in his laboratory as a routine measure on the admission of the patient, in others by his assistant in the out-door department, in a few instances in which it had been done both in the out-door department and on admission to hospital, and in which the two readings did not coincide the latter reading only was entered in the tables. This test (by Col Chopra's technique) has for some time been done as a routine measure in the laboratory of the senior writer in all out-patient cases sent to the department for diagnosis, these results have also been entered.

Col Chopra considers that a positive reaction with both the strong serum and the dilute (1 in 10) constitutes a 'positive' result and indicates kala-azar, a positive reaction in the former and a negative or doubtful reaction in the latter constitutes a 'doubtful' result, whereas a negative result in both tests constitutes a 'negative' result and indicates that the patient is not suffering from kala-azar.

In the cases of Table III Col Chopra's test, done in his laboratory, was positive in 20 out of 21 cases in which it was carried out, and doubtful in the other, done in the senior writer's laboratory it was positive in each of the 20 cases in which it was carried out. In the cases of Table IV (controls) the test was entirely negative in 21 and doubtful in 4 cases. These results are highly satisfactory, as also were ours in these two groups. However, in the cases of Tables V and VI the results were quite different. In Table V (kala-azar) Col Chopra obtained 4 entirely negative results, 4 positive results and 7 doubtful results, and in Table VI (non-leishmania) 4 positive results and 6 doubtful ones. That is to say of the 25 tests in 4 the result was correct, in 8 it was incorrect and in 13 it was inconclusive. Whereas the senior writer's figures for the same test were 3 entirely negative results, 10 positive and 8 doubtful in Table V (kala-azar) and 2 negative, 14 positive and 6 doubtful in Table VI (non-leishmania). That is to say of the 43 tests 12 were correct, 17 incorrect

and 14 were inconclusive. It is thus apparent that these were a particularly difficult group of cases as by this test—which usually quite satisfactory—far more incorrect than correct results were obtained even when the test was carried out in Col Chopra's own laboratory. Our fears therefore that our unsatisfactory results were due to some special feature of our technique were unnecessary.

*Observations.* Examination of the tables brings out a certain number of points. Where the stronger dilutions of urea-stibamine are used the number of positive results was according to the order of the strength of the serum used but when we come to the 1 per cent urea-stibamine it will be seen that the strong serum gives fewer positive results than the 50 per cent serum and with the 0.5 per cent urea-stibamine the strong serum gives fewer positive results than both the 50 per cent and 25 per cent. Again with the undiluted 50 per cent and 25 per cent serum the number of positive results were fewest when 4 per cent urea-stibamine was added. This suggests that when either the serum or the urea-stibamine are in excess there is a re-resolution of the precipitate. The limits between which little or no re-resolution occurs would appear to be as follows:

With 5 mg of urea-stibamine not more than 0.25 c.c. serum and with 0.0625 of serum not more than 10 mg of urea-stibamine.

Col Chopra's dilution method in which he adds equal parts of 1 in 10 serum to 4 per cent urea-stibamine solution, is not within these limits. This may account for the somewhat irregular results obtained by this test, as the degree of mixing which under these circumstances is an important factor will vary in the hands of different observers and with the calibre of the tubes used.

#### *Comparison between the aldehyde and the antimony test readings*

Although these readings are not parallel throughout—for example, in Table III case 6 has a +++ aldehyde reaction, but only gives 11 positive antimony test readings whereas case 12 has only a + aldehyde reaction and yet gives 12 positive antimony readings—there is a distinct parallelism in the two sets of readings as the following Table VIII compiled from Tables III, IV and VI shows.

TABLE VIII

ALDEHYDE REACTION		ANTIMONY TEST, DONE IN THESE CASES	
Reading	Number showing this reading	Total number of positive readings in 16 different dilutions in each of these cases	Average number positive readings per case out of a total of 16 tests in each case
+++	18	266	14.8
++	2	21	10.5
+	5	49	9.8
(+)	27	193	7.1
±	15	72	4.8
(-)	2	10	5.0
-ive	6	5	0.8

## DISCUSSION

The antimony test—like the aldehyde test—is in no way a specific test. It merely demonstrates the presence of some 'quality' in the serum which is present both in the kala-azar and in certain other conditions but which, however, is present to a greater degree in advanced kala-azar than in any other disease (as far as experience in India shows). This 'quality' makes its appearance soon after the onset of the kala-azar and increases throughout the disease. (It is almost certainly the same quality in the serum which produces both the precipitations with urea-stibamine and the milky gelification with formalin.) The antimony test is designed to show the presence of this 'quality'. When strong serum is added to 4 per cent urea-stibamine the slightest trace of this 'quality' will be demonstrated, whereas when both the serum and the antimony compound are diluted a point is eventually reached when the presence of this 'quality' is only demonstrated if it is present in a very marked degree, such a degree as is only associated with kala-azar and does not occur in any other disease. It is these dilutions of the serum and antimony compound which are the important ones from a diagnostic point of view, as they are the critical dilutions which separate definite cases of kala-azar from doubtful cases. Beyond this point the test can only be used for supplying contributory evidence, although this evidence may be extremely valuable.

Now in our series of experiments the only dilutions which are up to this standard are D, H, and L, that is 12.5 per cent serum with 1, 2, and 4 per cent urea-stibamine respectively. But in only 12, 15, and 15, respectively, of the cases of advanced kala-azar (Table III) gave positive results with these dilutions, the practical value of these readings would be very limited.

Our observations show that, within certain limits referred to above—the greater the concentration of both the serum and the antimony solution, the greater the amount of precipitate formed, but that beyond this there is no special concentration of either the serum or the antimony which is more favourable than any other for the production of a precipitate, whether the serum being tested be from a patient suffering from kala-azar or from one suffering from a some other clinically similar condition.

It is apparent that the test should be looked upon as a quantitative, rather than a qualitative one and probably the best information would be obtained by performing it in a number of different dilutions, as we have done here, this, however, would be impracticable where a large number of patients have to be dealt with. It would obviously be much more satisfactory if the test could be made with a single combination of dilutions.

*Choice of dilutions.* Turning back to Table VII, counting a + as positive and anything less as negative, it will be seen that with each of some half dozen dilutions the correct diagnosis was made on 75 to 77 occasions. Judging the matter on this standard it would not appear to be a matter of very great importance which of these combinations of dilutions is used for performing the test.

If, however, only a single test is to be carried out it will be advisable to have three readings positive, negative and doubtful. Let us review the results again taking three readings. These are summarized in Table IX.

TABLE IX

Dilution (See Tables III to VI)	Number of correct results, either positive or negative	Number of incorrect results, either positive or negative	Number of doubtful results
A	62	24	14
B	65	20	15
C	60	8	32
D	58	25	17
E	55	22	23
F	59	19	22
G	59	8	33
H	60	19	21
I	46	14	40
J	61	18	21
K	58	7	35
L	57	15	28
M	32	6	62
N	48	8	44
O	52	5	43
P	57	11	32

It is apparent from this table that the most satisfactory results are obtained by using 25 per cent serum, whatever the dilution of the antimony solution. On the whole probably the most satisfactory is combination 'K', i.e., 25 per cent serum + 1 per cent urea-stibamine, and these happen to be the dilutions which also gave the most satisfactory results in Table VII.

*The technique.* To obtain this combination it is not necessary to make up the urea-stibamine solution and dilute serum solution separately, almost the same result will be achieved by adding 2 drops of serum from a broad-pointed

\* Combination 'K' consists of 0.5 c.c. of 25 per cent serum + 0.5 c.c. of 1 per cent urea stibamine, the final dilutions will thus be 1 in 8, and 0.5 per cent respectively. By adding 0.125 c.c. to 1 c.c. of 0.5 per cent urea stibamine the dilution of the serum will be 1 in 9 and the urea stibamine 0.44 per cent.

dropper—16 drops to the cubic centimetre—to 1 c.c. of a 0.5 per cent urea-stibamine solution. If tubes of the same calibre are used the same criterion of positivity could be employed—namely the complete blurring of the window outline when the tube is held against the light—and the result should be considered 'doubtful' if there is more clouding than occurs in the distilled-water plus-serum control which should be put up at the same time.

This method would be far simpler to perform than the serum dilution method advocated by Col. Chopra. The latter can scarcely be classed as a bed-side method, as the accurate diluting of serum though an extremely simple matter to the laboratory worker would present certain difficulties to the average clinician.

That this combination of dilutions when prepared by the method we followed gives the most satisfactory results has been demonstrated above, it now remains to be seen whether the slight modification which we have suggested to simplify the test will affect its utility. To show this the test will have to be made in a large series of cases. This is at present being done, the results so far obtained have been highly satisfactory.

#### SUMMARY

For the clinical application of the antimony test for kala-azar different observers have suggested different dilutions of both the antimony compound and the serum. The present writers have carried out a series of experiments with different dilutions of both serum and antimony compound in order to find out which combination of dilutions gives the most specific results.

For this purpose 4 groups of 25 patients each were selected, the first two groups consisted of patients in whom a definite diagnosis had been made by the aldehyde test, 25 kala-azar patients and 25 controls, and the other two groups consisted of cases in which the aldehyde test gave an indeterminate result, of these again 25 were kala-azar patients and 25 culturally proved not to be kala-azar.

The most satisfactory results were obtained by the addition of 25 per cent serum to either 4 per cent, 2 per cent, 1 per cent or 0.5 per cent urea-stibamine.

By adding this strength of serum to 1 per cent antimony compound the readings in the series were 58 correct, 7 incorrect and 35 doubtful, this is a satisfactory result.

Almost the same mixture of antimony compound and serum can be obtained by adding 2 drops (0.125 c.c.) of serum to 1 c.c. of 0.5 per cent urea-stibamine, this being a more practical method is suggested for the clinical application of the test.

Preliminary clinical trial has given satisfactory results.



# STUDIES IN DISINFECTION AND STERILIZATION

## Part I

### GERMICIDAL PROPERTIES OF CERTAIN INDIAN ESSENTIAL OILS

BY

P. K. DEB

AND

A. SUBRAHMANYAN

*Department of Biochemistry, Indian Institute of Science, Bangalore*

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The antiseptic properties of essential oils were first established by Jalen de la Croix (1881) Chamberland(1) (1888) and Fiedenreich(2) (1889) who found that bacteria were killed on exposure to vapours of essential oils for periods varying from a few minutes to less than an hour. But the quantitative study of the relative bactericidal efficiencies began only in 1910 when Martinale(3) determined the phenol coefficients of a large number of essential oils. Since then several workers have dealt with the subject. Greig Smith(4) investigated the germicidal power of eucalyptus oils. Schobl and Kusani(5) established the inhibitory effect of the volatile constituents of a number of oils on the growth of tubercle bacilli on agar media. Dyche-Teague(6) and later Bryant(7) examined a large number of commercial perfumes and found that many of them possessed antiseptic powers. A further advance was made by Penfold and Grant(8) who studied the antiseptic properties of Australian essential oils and their constituents. They determined the phenol coefficients of a large number of terpene derivatives and observed that many of them gave high values. They also showed that in commercial preparations of concentrated disinfectants large quantities of active agents were wasted. Morel and Rochaix(9) tried the action of a large number of vegetable oils in both liquid and vaporized states, on various pathogenic germs and showed that the antiseptic action of oils varied considerably and that some germs were killed more readily than others.



Rideal, Rideal and Sciver(10) showed that the surface activities of essential oils were generally closely related to their germicidal properties

According to Wilhelm Dreyfus(11) a useful preparation of germicide should (1) possess high germicidal power, (2) form homogeneous emulsion with water, (3) be free from sediments, (4) be non-poisonous, non-corrosive and non-toxic to higher organisms, (5) possess deodorizing power, (6) be stable in presence of organic matter, (7) dissolve and penetrate grease and fat, and (8) be cheap. Essential oil germicides have been found to satisfy most of the above requirements.

Toxicity and corrosiveness are the greatest drawbacks of coal-tar disinfectants. Accidents arising from the use of such preparations are usually due to phenols, cresols and their derivatives. A large number of essential oils possess high antiseptic properties, but are relatively non-toxic to human beings. Although many of them are more costly than coal-tar disinfectants, it should still be possible to select some which are (a) more powerful germicides than the latter, and (b) obtainable comparatively cheaply.

Because of their high germicidal power essential oils have, during recent years found considerable application as disinfectant medicines and in surgery. Their use has been recommended (Penfold and Grant, *loc cit*) in the preservation of antisera and similar products which are, at present, prevented from bacterial decomposition by addition of phenol and mixed cresols. Cresols, in particular, gradually destroy active principles present in antisera, cause considerable loss to the manufacturer and necessitate an increase of dosage to be administered to patients. Essential oils have been successfully used in healing septic war wounds. Terpeneless oil of lavender has been successfully used in treating gangrenous wounds, in certain nasal diseases, in ulcers and in syphilitic degeneration of the mouth and nose where mercurial treatment had failed.

These valuable properties of essential oils led us to think that additional facts contributing to our present knowledge about them might be of considerable assistance in directing the search for more efficient but less costly antiseptics.

In India, the raw materials for essential oils are obtainable in abundance and recently, much attention has been paid to extraction of these oils cheaply on a large scale. During the extraction of essential oils, large quantities of sesquiterpenes are obtained which, at present, are regarded as waste products. It is of considerable interest and importance to determine whether these products can be used as disinfectants either by themselves, or as derivatives after introducing in their molecules active groups which will enhance their germicidal power.

An investigation was, therefore undertaken with the object of determining (1) the suitability of Indian essential oils for use as germicides in place of coal-tar disinfectants, (2) whether sesquiterpenes can be utilized as disinfectants.

This paper will present the germicidal values of a number of Indian essential oils and sesquiterpene derivatives while further discussion as to whether any of them can be used as disinfectant in place of coal tar products is reserved for a subsequent communication

### EXPERIMENTAL

Since essential oils are insoluble in water and emulsified disinfectants are much more reactive than dissolved ones emulsions of the different oils were used for determining their germicidal properties. Since the efficiency of an emulsion depends on the use of correct proportions of oil, water and emulsifier and the mode of preparation attempts were made to standardize the techniques of preparing the emulsions. Soaps were not used because they contained, invariably impurities which vitiated the results to considerable extent. Pure sodium oleate was used as the emulsifying agent. There was some difficulty in the earlier stages owing to the oleate solutions undergoing partial hydrolysis, on standing with the result that oleic acid and an insoluble acid soap separated out and affected the stability of the emulsions but it was overcome by addition of a little sodium carbonate which stopped the hydrolysis of sodium oleate.

To determine whether the oleate carbonate mixture was also a better emulsifier than the oleate alone a number of emulsions were prepared by adding in each case 1 cc of oil (*Cymbopogon Martini* Stapf Sofia) to 99 cc of solutions of oleate alone and oleate with carbonate and their respective stabilities tested at intervals of 12 hours. Concentrations of free alkali were not increased further as excess had been shown to have deleterious action on the emulsifying power of soap (12). It was observed that while an emulsion prepared with 4 per cent oleate was not stable for more than 12 hours, one made with a mixture containing 1 per cent each of oleate and carbonate remained indefinitely stable.

Martin Frobisher, Jr (13), studied the influence of surface tension reducers on the germicidal activity of phenol and hexylresorcinol observed that while the addition of optimum amount of soap led to maximum germicidal value, an excess gave a much lower value. He stated that excess of soap coated the bacterial surface with a film of sodium oleate which protected the cells from contact with phenol. Addition of a liquid surface tension reducer, on the other hand, enhanced the germicidal activity as, in this case, the adsorbed substance, being liquid, was still miscible with phenol and opposed no solid barrier to its diffusion into the cell. To determine whether the concentration of soap had any influence on the emulsified disinfectants, a number of emulsions containing identical quantities of oil (1 per cent) but differing in the amounts of sodium oleate used to emulsify them were prepared and their bactericidal powers determined by the Rideal-Walker method using *B typhosus* as the test organism. It was observed that increasing amounts

of sodium oleate lowered the germicidal activity as will appear from the following Table —

TABLE I

Concentration of disinfectant	1 PER CENT OLEATE + 1 PER CENT $\text{Na}_2\text{CO}_3$						2 PER CENT OLEATE + 1 PER CENT $\text{Na}_2\text{CO}_3$						3 PER CENT OLEATE + 1 PER CENT $\text{Na}_2\text{CO}_3$					
	Minutes exposure						Minutes exposure						Minutes exposure					
	2½	5	7½	10	12½	15	2½	5	7½	10	12½	15	2½	5	7½	10	12½	15
1 900	+	—	—	—	—	—	+	+	—	—	—	—	+	—	+	—	—	—
1 1000	+	—	—	—	—	—	+	+	+	—	—	—	—	+	+	+	—	—
1 1100	+	+	—	—	—	—	+	+	+	—	—	—	+	+	+	+	+	+
1 1200	+	+	+	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+
1 1300	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ indicates growth, — no growth

With lower concentrations of oleate no stable emulsion was obtained

It is possible that in a concentrated solution of the oleate, (a) the essential oil was not dispersed so evenly as when the solution contained the optimum amount of soap with the result that the active surface of the germicide was greatly lowered, (b) the excess of soap might have been adsorbed on the bacterial cells as observed in the case of phenol and hexylresorcinol or (c) the thickness of the interfacial layer of soap between the oil-water boundary was so much that the oil could not diffuse through it and come into contact with the bacterial cells. The results of the foregoing experiment may find application in the preparation of disinfectants emulsified with soap.

To determine whether persistent contact with a high concentration of oleate affected the disinfecting property even after dilution 5 and 1 g. lots of the oil were emulsified with five and one per cent solutions respectively of the oleate, and then disinfecting properties compared after diluting the concentrated solution five times so as to make their compositions identical. On comparing the germicidal strengths of the two emulsions it was observed that the original 1 per cent emulsion was much more active than the one obtained by diluting the 5 per cent emulsion.

The difference in activity was probably due to that between the degrees of dispersion of the two emulsions. In the diluted emulsion the oil had more surface for dispersion than in the concentrated one. By dilution of the latter with water, the degree of dispersion was not much changed; the sizes of many of the individual particles remaining the same as in the concentrated solution. The foregoing

observations are in accordance with those of Penfold and Grant (*loc. cit.*) on Australian essential oils.

In the present investigation 1 per cent emulsions of oils in solutions containing 1 per cent each of sodium oleate and sodium carbonate were used. In a few cases however the concentration of soap in the emulsifying mixture had to be increased slightly since one per cent soap did not give a stable emulsion.

It was observed that all the emulsions were stable and could be diluted with water in all proportions without any separation of oil. Trials showed however that on prolonged keeping there was some decrease in germicidal power. Results of observations on the effect of keeping the oils are shown in the following Table.

TABLE II

Name of oil	Phenol coefficient of fresh emulsion	Age of emulsion in months	Phenol coefficient after keeping
Cinger grass oil	10.0	1	9.0
Indian Cubel oil	7.0	1	7.0
Camphor leaf oil	1.0	11	1.2
1:4 Cineol	8.1	1	7.5
Cineol	5.9	1	1.7
Tenueel oil	11.0	11	10.0
Cardamom oil	10.0	11	Coefficient falls very quickly

#### *Phenol coefficients of essential oils and sesquiterpene derivatives*

Commoner oils and sesquiterpenes which can be obtained cheaply and in abundance were chosen for the trials. They were all isolated and purified in the Organic Chemistry Department of the Institute and were obtained through the courtesy of Mr. B. Sanjiva Rao to whom the authors' thanks are due.

The Rideal-Walker method was followed rigidly using *B. typhosus* (Hopkins) as the test organism. The following dilutions were first tested 1:100, 1:300, 1:500, 1:700, 1:900 and from the results it was possible to ascertain the limits within which the desired dilution should be. Smaller dilutions were then made in the following scale — 1:70 to 1:160 by differences of 10, 1:160 to 1:200 by differences of 20, 1:200 to 1:400 by differences of 25, 1:400 to 1:900 by differences of 50. If the required concentration was found to be above 1:900, higher dilutions were made by differences of 100 up to 1:1800 and higher of 200 up to 1:3200. Action of the prepared dilutions was then tried on the organism and from the results the dilutions required to kill the organism at the

end of five minutes were obtained. The corresponding dilutions of phenol were next determined. The results are given in the following Table —

TABLE III

Oil or derivative	Main constituents	Phenol coefficient
<i>Cymbopogon Gaesius</i> (Kachi grass oil)	Geraniol, Perillic alcohol, Terpenes	3.6
<i>Cymbopogon coloratus</i> (Botha grass oil)	Sesquiterpenes	1.5
<i>Cymbopogon flaxnosus</i> (Lemon grass oil)	Citral 70 — 85 per cent	17.0
<i>Cymbopogon Martini</i> Stapf Motia (Palmarosa oil)	Geraniol 75 — 95 per cent	14.0
<i>Cymbopogon Martini</i> Stapf sofia (Ginger grass oil)	Geraniol	10.0
Indian Cubeb oil		7.0
Camphor oil	Hydrocarbons, safrol, and camphor	6.2
Camphor leaf oil	Cineol, Terpeneol	4.0
Cardamom oil	Terpeneol, Broneol, and Cineol	10.0
Fennel oil	Anethole	13.0
Clove oil	Eugenol	5.5
Longifolene	Sesquiterpene hydrocarbon	less than 1.0
Santalene	Do	Do
B—Santalol	Sesquiterpene alcohol	1.6
Trichloro derivative of a sesquiterpene		less than 1.0
Nitro derivative of a sesquiterpene		Do
<i>Hardwickia Pinata</i> ..	Sesquiterpene	Do

### Discussion of the Results

The phenol coefficients of the oils varied from 1.5 to 17.0. Of the three oils, *Cymbopogon flaxnosus*, *Cymbopogon Martini* Stapf sofia and *Cymbopogon Martini* Stapf Motia which had the highest values, the last is somewhat costly and should be left out of consideration. The other two oils are fairly cheap and hold out prospect of successful application. Sesquiterpenes were all inactive. Even the introduction of three chlorine atoms in the molecule did not enhance their germicidal values to any appreciable extent.

An investigation to determine whether disinfectants prepared with *Cymbopogon flarnosus* and *Cymbopogon Martini* Stapf can be used successfully in place of other disinfectants on the market, is in progress and will form the subject of a later communication.

The authors' thanks are due to Dr. R. V. Norris for his keen interest in the progress of the investigation.

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minutes' (6) or 'ten minutes after hæmolysis is complete in the front row tube of the normal serum control' (7) these reactions are missed. In the writers' procedure the final incubation is done for half an hour as in determining the M H D of the complement.

That these irregular reactions are best classed with the doubtful reactions is shown by a comparison with the doubtful cases in the following summary —

#### 100 Doubtful cases ( $\pm$ )

Presumption of syphilis	{ Early	24
	{ Treated	9
	{ Untreated	6
No presumption of syphilis		5
Indefinite		56
		<hr/> 100

#### 100 Irregular cases

Reaction could be read as —

		+	+	+	-	
Presumption of syphilis	{ Early	3	8	17	=	28
	{ Treated	1	1	6	=	8
	{ Untreated	0	4	5	=	9
No presumption of syphilis		0	1	0	=	1
Indefinite		1	11	39	=	51
		<hr/> 5	<hr/> 28	<hr/> 67		<hr/> 100

Each series consists of unselected cases taken serially. A few unaccompanied by histories have been discarded. A close correspondence between the clinical histories and the serological findings is apparent.

3. *In the absence of a standardized red cell suspension the M H D of the complement, obtained on different days, is not comparable.*

Red cells collected and washed apparently under identical conditions do not yield a deposit of the same density always. A 3 per cent suspension (method IV) made from the deposit, consequently, differs in density from day to day. The M H D of the complement, therefore, is not comparable on different days.

When tested colorimetrically the difference in density of the emulsion may amount to 20 to 30 per cent on different days. The M H D obtained by using a suspension of density X is less than the one obtained by using a suspension of density  $X + \frac{1}{3}X$ . The denser is the suspension the bigger is the M H D.

In the technique of method IV the various constituents happen to be so adjusted that the antigen destroys exactly 1 M.H.D. of the complement determined by using a suspension of red blood cells of an optimum density. When the density of the suspension is greater than the optimum the M.H.D. of the complement obtained is higher than it should be with the result that the antigen cannot destroy 1 M.H.D. An excess of complement is left which makes itself visible in lack of correspondence between the two rows of tubes used in titrating the complement with and without the antigen. The tubes containing the antigen indicate a higher titre of the complement.

If regardless of the lack of correspondence the M.H.D. is taken from the tubes containing complement alone in the test proper there would be an excess of complement over the standard amount for the reason detailed above. The allowance made for the antigen will be 1 M.H.D. of the complement. As a matter of fact the complement destroyed by the antigen will be less than 1 M.H.D. This excess generally trivial may at times be 1, 3 M.H.D. an amount which will easily convert a  $+$ ,  $+$ ,  $+$ ,  $+$ , or  $+$  reaction into a  $+$ ,  $+$ ,  $+$ ,  $+$  or  $+$  reaction respectively.

In case the variation in the density of the suspension lies in opposite directions on two different occasions the difference in M.H.D. may easily amount to 2/3 M.H.D. of the complement.

Attention appears to have been drawn to this important adjustment of the hemolytic system long ago (8). In the usual works of reference and publications in English however one does not find any details.

The writers have found that the density of the red cell suspension, for the requirements of the method IV of the British Medical Research Council is optimum when 7 drops of a 6 per cent suspension, delivered from a Wright's pipette, with the point fitting hole No. 58 of the standard wire gauge, give a direct reading of 51 on Sahli's haemoglobinometer. Such drops measure 0.02 c.c. each.

A suspension giving a higher reading can be adjusted to give the required figure by dilution.

Example. A suspension gives a reading of 62. Total volume is 100 c.c.

$$\begin{aligned} 62 \quad 51 \quad 100 \quad X \quad X &= \frac{62 \times 100}{51} \\ &= 121.56 \\ &= 121.6 \text{ approx} \end{aligned}$$

An increase of volume to 121.6 c.c. will reduce the reading to 51.

A suspension giving a lower reading can be made to give a higher reading by the addition of more of the deposit and then adjusted as detailed above.

Since adopting this method of standardizing the red cell suspension the writers have generally found a perfect correspondence between the two rows of tubes used in titrating the complement with and without the antigen. This fact, incidentally,



emphasizes the importance of determining the M H D of the complement in the presence of the antigen. Any lack of adjustment is made visible by a lack of correspondence between the tubes of the two rows.

Regarding the normal limits of variation of the colour index of the sheep, as affected by age and environment, the writers have no knowledge. The observations leading to the findings relating to the standardizing of the red cell suspension were made between May and August 1929, the monsoon season of Burma. Meteorological conditions in this season are practically constant. Heat is tolerable. Green grass for the animals is abundant.

The sheep kept for blood in the Pasteur Institute of Burma are three Calcutta sheep of uncertain ages. They are bled in rotation and about 20 c.c. of blood is removed. The Wassermann reaction is done twice a week. The animals feed well and look well nourished and healthy.

Under different conditions of climate, food supply and altitude, etc., the colour index might be affected appreciably. It should be always possible, however, to so modify the standard as to obtain a suspension of optimum density, i.e., a suspension which will yield an M H D of the complement which is exactly neutralized by the antigen.

4 *Under certain conditions, not fully understood, sometimes the antigen does not destroy one full M H D of the complement as obtained with a standardized red cell suspension.*

At times, in the titration of the complement with and without the antigen, the row of tubes containing the complement alone indicates a titre, say, 1 in 40, while the row containing the antigen plus the complement twice as strong indicates a titre of 1 in 60. Obviously the antigen has not destroyed 1 M H D of the complement but only  $1/2$  M H D.

The writers had thought they had abolished all lack of correspondence by standardizing the red cell suspension when it appeared again. Evidently some other factor is also concerned.

By a process of exclusion the writers have come to believe that the complement alone is responsible for this altered relationship between the antigen and the complement.

The altered relationship results in (1) an excess of the functioning dose of the complement in the test proper, and (2) a corresponding deficiency in the dose of the complement in the special serum control.

So far, the practice of the writers has been to increase proportionately the complement in the special serum controls to avoid false weak positive (+) and doubtful ( $\pm$ ) reactions while keeping the standard dose of the complement used in the Institute unaltered. In doing so, however, there is a risk of missing a certain number of weak positive and doubtful reactions. Now it is proposed to keep the functioning dose of the complement constant, i.e., when, as in the instance given,

the antigen destroys only  $1/2$  M H D of the complement, instead of 1 M H D. The dose for the test would be

Test proper	$1\frac{1}{2}$ M H D and 2 M H D
Serum controls	$2\frac{1}{2}$ M H D and $1\frac{1}{2}$ M H D

Instead of

Test proper	1 M H D and $2\frac{1}{2}$ M H D
Serum controls	$2\frac{1}{2}$ M H D and $1\frac{1}{2}$ M H D

Whether a complement which is destroyed less by an antigen fixes the reagin normally, the writers have not determined. Non anti-complementary antigen (i.e., used in non anti-complementary doses) however, are as much in use as the anti-complementary ones.

5. *The real and doubtful reactions can only be detected by a small dose of the complement*

*Specificity* (using the term for convenience only) of a Wassermann reaction varies with the antigen employed. Having selected a particular antigen (Fildes and McIntosh in method IV) it is not possible to increase or decrease the specificity by varying the relative proportions of the antigen and the complement. If that were so, one would sometimes find stronger reactions occurring with bigger doses of the complement.

*Sensitiveness* of the reaction on the other hand varies inversely with the dose of the complement. To say then that 'the inclusion of still smaller amount (e.g., 2 M H D or 3 M H D) introduces the fallacy of non specific deviation with normal sera' (9) is only to create confusion of ideas. What is really meant is that some conditions are likely to produce a semblance of a positive reaction with a small dose of the complement. This semblance, the writers believe, is only produced by a *moderately high* anti-complementary titre of a serum, and stands revealed as such when a proper serum control giving an indication of the anti-complementary titre of the serum is put up.

The semblance produced is only of a  $\pm$  or a  $+$  reaction which depend for their existence on 1 M H D or less than 1 M H D of the complement. When a liberal dose of the complement is used these reactions naturally do not occur. The semblance is abolished, *but so are also the true weak reactions*. The real remedy, then, is not a liberal dose of the complement but the proper control indicating the anti-complementary titre of a moderately high titre serum (a titre lying between  $1/2$  M H D and 2 M H D of the complement when using method IV).

Certain well known clinical entities and conditions are known to give a true positive Wassermann reaction. The workers who advocate liberal doses of the complement have never produced any figures to show that this reaction disappears with bigger doses of the complement *leaving the true weak reaction of syphilis intact*. It is only the semblance which is raised or lowered by decreasing or increasing the complement, not the specificity.

Other things being equal, a method employing a small dose of the complement is more sensitive and therefore more valuable for detecting syphilis than the

one employing a large dose At the Pasteur Institute of Burma Rangoon, 2½ M H D and 4 M H D are used instead of 3 M H D and 5 M H D as recommended in method IV The following summary gives the reactions and associated clinical condition of the 1,000 sera —

			++ or +	±	—
Presumption of syphilis	Early (primary)	70	30	14	26
	Treated	102	39	14	49
	Untreated (or treated with indigenous medicine)	428	318	29	81
No presumption of syphilis		59	0	4	55
Indefinite		441	141	33	164
		1,000	531	94	375

These cases were taken serially discarding some which were unaccompanied by histories

#### 6 The coloured sera

A record was kept of the physical condition of the sera received for the Wassermann reaction The following summary gives then reactions —

#### 500 coloured sera

	TOTAL	++ or +	±	—	Anti complementary
1 Hæmolysed Red, brown or pink	329	162	40	108	19
2 Bile tinged yellow or green	30	21	2	6	1
3 Chylous	85	67	5	8	5
4 Turbid with growth	56	44	2	6	4

#### 1,000 mixed sera for comparison

++ or +	±	—
531	94	375

The following points emerge —

(1) Hæmolysed sera give the various reactions in practically the same proportion as the mixed sera, the general prejudice against them, therefore, is unfounded There is, however, one serious flaw in a red serum Without employing a special serum control (consisting of, (i) the serum under test, (ii) antigen, (iii) red cell suspension, (iv) saline instead of complement) it is very difficult to decide whether a reaction is.—

3 M H D	5 M H D	Reading
I	I	
or L trace	L trace	(irregular)

(ii) Bile tinged sera give a greater proportion of positive reactions than the mixed sera. The fact, however, is not necessarily associated with the mere presence of bile in the serum. The negative reactions are as clear as with the normal sera. Syphilis and its remedie (particularly the indigenous drugs and the indigenous system of medication which lack standardization both in dosage and in administration) are perhaps responsible for the jaundice in many cases. The defect, moreover, cannot be overcome. The serum from a jaundice must be done if warranted by clinical observations.

(iii) Ordinary chylous sera give a high positive figure. The defect, however, can be overcome, part from the existence of a fibrin or similar cause. Blood taken in the morning before food will yield a clear serum as a rule.

(iv) Sera turbid on account of contamination and bacterial growth give the highest positive figure. How far the positiveness is due to syphilis and how far to the extraneous agents in the growth the writers have not determined. This defect again can be and should be overcome whenever possible. Excluding septicemia, it is generally blood collected in drops from fingers and toes which gets heavily contaminated.

To sum up, positive reactions given by slightly haemolysed sera and bile tinged sera are to be taken as positive, while positive reactions given by deep red sera, chylous sera or grossly contaminated sera should only be taken as doubtful reactions and repeated with fresh sera.

#### 7. *Certain associated considerations*

(i) The antigen. The antigen does not appear to change at all, with keeping, even under tropical conditions as long as the evaporation is prevented. The writers' antigen has been prepared according to the method of Fildes and McIntosh, from fresh human hearts of young and healthy subjects. All the hearts so far used were obtained from jails after executions. The senior writer had seen all the subjects previously. This keeping power of the antigen is important inasmuch as both the complement and the red cell suspension are dependent on the antigen with regard to a standard dose. 1 M.H.D. of the complement obtained with a red cell suspension of an optimum density is exactly neutralized by the antigen used in a constant dose.

(ii) Opalescence resulting from a mixture of certain sera with the antigen. In the test of some sera while the lysis is quite complete in all the four tubes (2 test proper, 2 serum controls), in the tubes of the test proper a fine opalescence is observed. No connection has been observed between this opalescence and the precipitation phenomenon of the Kahn test done on the same sera. The opalescence does not signify a doubtful reaction.

(iii) A negative or a doubtful reaction becoming positive after a provocation injection. The number of such reactions is not inconsiderable. They are not to be regarded as doubtful, once a positive result has been obtained, simply because very soon before the positive reaction they gave a doubtful or a negative reaction.

## SUMMARY

1 In doing a Wassermann test with an anti-complementary antigen a serum control containing the functioning dose of the complement is essential. Without such control some negative but slightly anti-complementary sera will be returned as  $\pm$  or  $+$ .

2 Irregular reactions occur with a constant frequency. They are best classed with doubtful reactions, whatever the degree of inhibition of lysis may be.

3 Red cell suspension is not always of a constant density. It can be standardized colorimetrically. The M H D of the complement determined with an unstandardized suspension is not comparable.

4 Under some conditions not fully understood, sometimes the antigen does not destroy one full M H D of complement as determined with a standardized red cell suspension. The allowance for the antigen then should only be the amount actually destroyed, not 1 M H D.

5 Of coloured sera positive reactions given by slightly hæmolysed sera and bile tinged sera are to be accepted, while positive reactions given by deep red sera, chylous sera or grossly contaminated sera are to be looked upon as  $\pm$  and repeated with fresh sera.

6 Weak and doubtful reactions can only be detected by using a small dose of the complement. The concomittent difficulty caused by sera of a moderately high anticomplementary titre can be effectually eliminated by using the serum control containing the functioning dose of the complement.

7 Antigen does not deteriorate with keeping. A fine opalescence given by some sera, in the absence of any inhibition of lysis, is not a doubtful reaction.

8 The diagnostic value of a positive reaction is not decreased by the fact that a provocation injection was necessary to bring it out.

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# DISSOLVED OXYGEN IN RELATION TO ANOPHELES BREEDING

BY

M O T IYENGAR B.A. 1928

Entomologist Bengal Public Health Department Calcutta School of  
Tropical Medicine and Hygiene

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CONSIDERABLE interest is now centred on the physical characters of the water of mosquito breeding places and much detailed work has been done in different parts of the world with regard to the hydrogen ion concentration and the amount of dissolved oxygen of dissolved nitrites, dissolved carbon dioxide etc present in the water of different types of breeding places Senior-White's(1926) has been the most important work carried out on this subject in India

The present investigations were undertaken to study the range of variation in the amount of dissolved oxygen among one particular type of breeding places, namely, of stagnant water collections. The observations were carried out at Sonarpur, 24-Parganas District, Bengal on ponds and ditches of varying sizes containing stagnant fresh water. Six species of *Anopheles* breed in these stagnant ponds and ditches, namely, *A. barbirostris* van der Wulp, *A. hyrcanus* var *nigerimus* James, *A. fuliginosus* Giles, *A. minimus* var *varuna* Iyengar, *A. subpictus* Grassi and *A. pseudojamesi* Stuck & Chdy. The investigations were carried out to ascertain if the distribution and numerical prevalence of these six species in the different ponds were in any way related to the amount of dissolved oxygen in the water. Estimations of the dissolved oxygen in the water of 332 breeding places in this area were carried out during November 1925, but results have unfortunately been greatly delayed in preparing for publication.

*Technique* The collection of water for the estimation of oxygen was carried out with considerable precaution to avoid all possibilities of absorption of oxygen

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\* Senior-White, R. Physical Factors in Mosquito Ecology, *Bull Ent Res*, Vol XVI, Part 3, pp 187-248, 1926

during the process of collection. The details of the technique of collection are given in Appendix I. The samples of water from the ponds were collected in the morning hours between 8 and 10 a.m., and this was done with a view to minimize the possibilities of error due to collection of the samples at different periods of the day. The importance of this procedure will be discussed at a later part of this paper. Side by side with the collection of water for the estimation of oxygen, the species of *Anopheles* breeding in the water and their numerical prevalence were also recorded.

The estimation of dissolved oxygen was carried out by Winkler's method with slight modifications. Details of the technique are given in Appendix I. It has been stated that the Winkler method is not reliable in foul waters with heavy nitrite contents, since the liberation of iodine by the oxygen could also be produced by the nitrites dissolved in the water, with the result that the estimations would not be indicative of the actual amount of oxygen dissolved in the water. But it may be pointed out that none of the ponds and ditches in the series discussed in the present paper was subject to any sewage pollution. To be sure that there were no nitrites in the water such as would affect the accuracy of the Winkler test in the estimation of dissolved oxygen, a series of test examinations of water from ponds and ditches in the present series were carried out for the presence of nitrites and it was found that none of them had even a trace of nitrites. These test examinations were carried by Mr. Narendranath Chatterjee, Assistant Analyst, Bengal Public Health Laboratory, and to him, the writer is much indebted for his kind help. The results of these test examinations show that there is no justification for doubting the accuracy of the Winkler test in the estimation of the dissolved oxygen content of the water of the present series of ponds and ditches.

Out of 332 ponds and ditches examined, the following results show the relative frequency of the varying degrees of dissolved oxygen in the water.

*Relative frequency of dissolved oxygen concentration in ponds and ditches in Sonarpur*

DISSOLVED OXYGEN IN MILLIGRAMMES PER LITRE							
TOTAL	0—1 mg	1—2 mg	2—3 mg	3—4 mg	4—5 mg	5—6 mg	6—7 mg
332	38	101	71	61	47	10	4
Percentage	11.1	30.4	21.4	18.4	14.1	3.0	1.2

CHART I

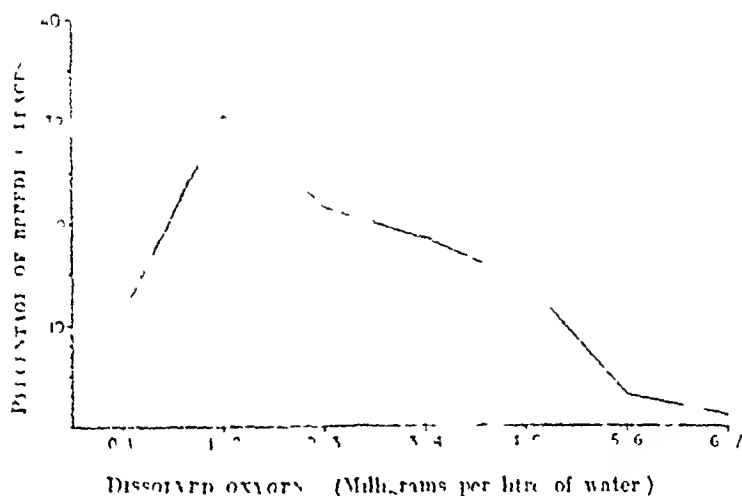


Chart I graphically represents the relative frequency of the concentration of dissolved oxygen in ponds in Sonarpur. The dissolved oxygen content of the major proportion of the breeding places in this area ranged between 1 to 3 mg per litre of water. Over 30 per cent of the breeding places had 1—2 mg of oxygen per litre, 21 per cent had 2—3 mg per litre, 18 per cent had 3—4 mg and 14 per cent had 4—5 mg. Only a very small number of breeding places had an oxygen content of over 5 mg per litre, the highest oxygen figure recorded in this series is 6.36 mg per litre.

The frequency of occurrence and the intensity of breeding of the six species of *Anopheles* in waters with varying concentrations of dissolved oxygen is discussed below. Two charts are drawn up for each of the six species of *Anopheles* investigated. The first chart shows the percentage of occurrence in the different grades of dissolved oxygen concentration. The second chart shows the intensity of breeding as shown by the average number of larvæ per breeding place in the different grades of dissolved oxygen concentration. The number of observations on the breeding places of these species both as regards the oxygen concentration as well as the numerical prevalence of the larval anopheles is sufficiently large to warrant discussion. This is so for each of the varying degrees of oxygen concentration between 0 and 5 mg grade. But in the case of the two last grades namely 5 to 6 mg and 6 to 7 mg, the number of observations made is far too small to be sufficiently representative, being only 10 and 4 respectively. As such, any conclusions regarding the frequency of the species of *Anopheles* or of their numerical prevalence in these two grades of oxygen concentration are not reliable since they are based on a very small number of observations. These two grades have therefore been omitted from the charts, but the details of the records are given in Appendix II. It may be pointed out,



however, that the number of observations of these two grades of oxygen concentration namely 5 to 6 and 6 to 7 mg, could not have been increased since their proportion to the total number has been found to be very small

CHART 2

*Anopheles barbirostris* in relation to dissolved oxygen

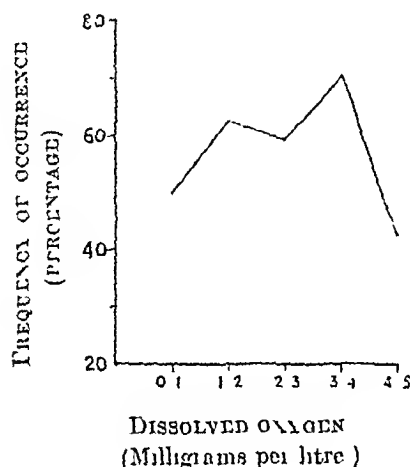
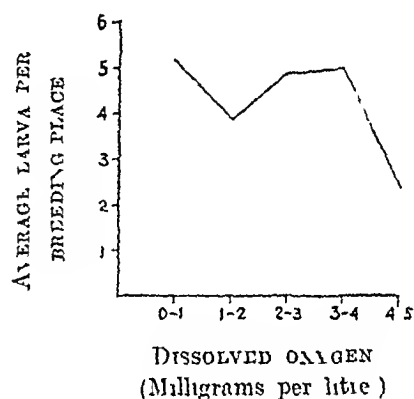


CHART 3



*Anopheles barbirostris* occurs in all grades of oxygen concentration. It is particularly common up to the 4 mg grade (Chart 2). As regards numerical prevalence of larvæ, there is no appreciable variation in the number in the different concentrations of dissolved oxygen (Chart 3). It seems that both as regards frequency of occurrence and numerical prevalence, *A. barbirostris* is not appreciably influenced by the degree of oxygen concentration.

CHART 4

*Anopheles hyrcanus* in relation to dissolved oxygen

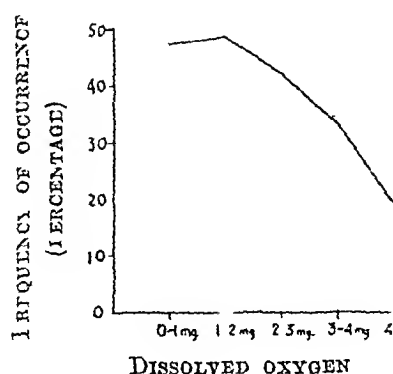
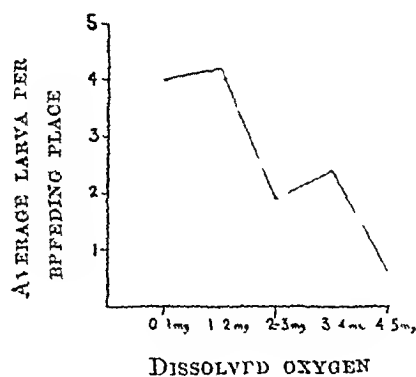


CHART 5



*Anopheles hyrcanus* occurs in all grades of dissolved oxygen concentration (Chart 4), its frequency is highest in the region of 1—2 mg and it steadily

diminishes with increasing oxygen concentration. The same tendency is noticed in regard to the numerical prevalence of the species (Chart 5).

CHART 6

*Anopheles fuliginosus* in relation to dissolved oxygen

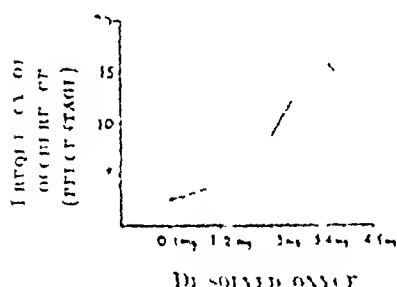
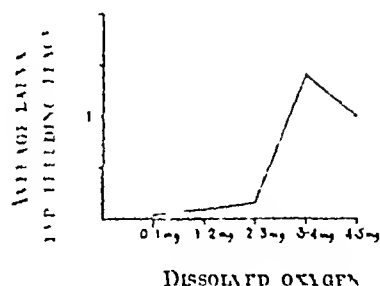


CHART 7



*Anopheles fuliginosus* shows a gradual rise in frequency with a rise in the oxygen concentration and the curve reaches its highest level in the region of 3—4 mg after which it declines (Chart 6). The average larva curve of this species which shows the intensity of breeding in each breeding place also exhibits the same tendency (Chart 7).

CHART 8

*Anopheles varuna* in relation to dissolved oxygen

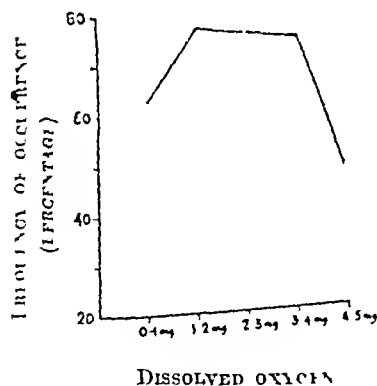
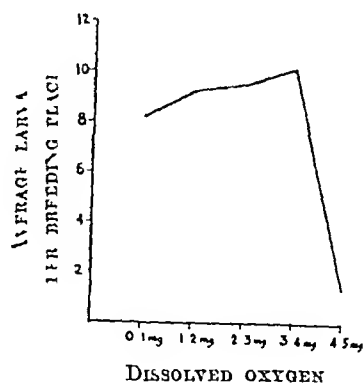


CHART 9



*Anopheles minimus* var. *varuna* is a very common species in the Sonarpur area. It has been found to breed in all grades of oxygen concentration and is particularly common in the 1—4 mg grades. It shows a tendency to decline slightly in the higher grades of dissolved oxygen concentration (Chart 8). The intensity of breeding (Chart 9) corresponds to the frequency curve. This species has been

found to breed in ditches containing water absolutely free from dissolved oxygen and also in ponds with an oxygen content as high as 6.10 mg per litre

CHART 10

*Anopheles subpictus* in relation to dissolved oxygen

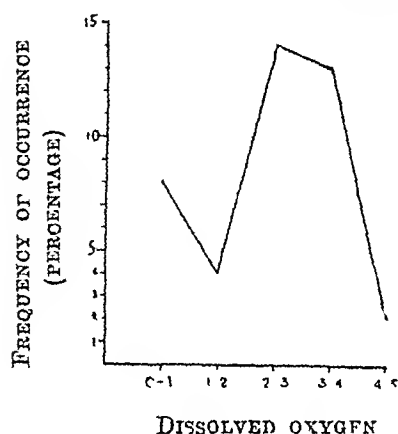
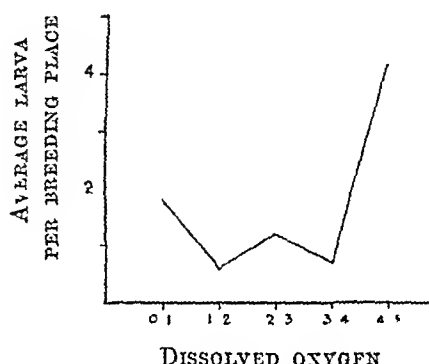


CHART 11



*Anopheles subpictus* occurs mostly in the 2—4 mg grades and declines sharply after that concentration (Chart 10). The average larva curve represented in Chart 11 does not show any relation to the frequency curve. The use of the former in the 4—5 mg grade is due to heavy breeding recorded from a single pond which has pushed up the average figure disproportionately.

CHART 12

*Anopheles pseudojamesi* in relation to dissolved oxygen

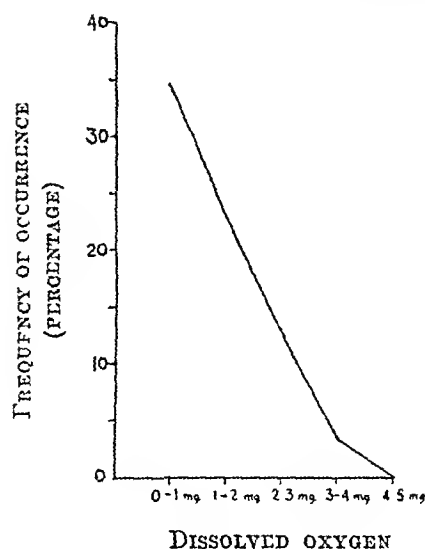
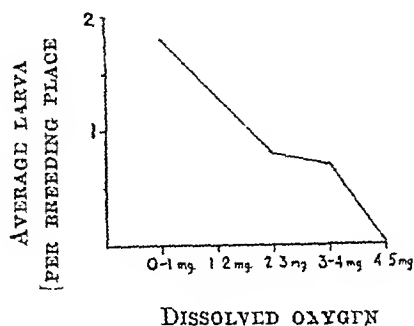


CHART 13



*Anopheles pseudojamesi* (syn *A. ramsayi* Covell) is most prevalent in the 0—1 mg concentration and from that high prevalence, it declines rapidly with an increase

in the oxygen concentration (Chart 12) The same tendency is noticed in the average larva curve (Chart 13) This species is usually associated with *Pistia stratiotes*, a floating plant which frequently covers up the entire surface of the ponds In ponds thickly covered over with *Pistia* the oxygen content is usually low

From the above discussed records it seems very doubtful if the oxygen content of the water has any direct relation with the prevalence of the different species of *Anopheles* except possibly in the case of *A. pseudojamesi* and *hyrcanus* The other species do not show any relation direct or inverse, with the concentration of dissolved oxygen in the water, either as regards frequency of occurrence or as regards numerical prevalence This is particularly true with regard to *A. barbinotris*, *varuna* and *subpictus* It is likely that a further examination of the records would show that even in the case of such of the species as do exhibit some relation to the concentration of oxygen in water, the relation is possibly due to an association with some types of aquatic vegetation

The range of distribution of each of the six species of *Anopheles* in the present series of observations is indeed very large They have been found to occur in nearly the entire range of oxygen concentration met with in the area *A. barbinotris* has been found from zero oxygen to 6.16 mg oxygen per litre, and this covers the entire range met with here *A. subpictus* ranges from zero oxygen to 6.10 mg oxygen *A. varuna* ranges from zero oxygen to 6.10 mg oxygen *A. hyrcanus* also has a very large range, from 0.16 mg to 6.46 mg *A. fuliginosus* ranges from 0.97 mg to 5.69 mg and *A. pseudojamesi* from 0.44 mg to 6.46 mg

#### Diurnal fluctuation of dissolved Oxygen Content of Pond water

The value of the so called 'oxygen toleration limits' of species of *Anopheles*, at any rate with regard to the species here investigated and the breeding places of the locality as discussed in the present paper, is very questionable This is especially so when it has been found during the course of these investigations that the oxygen content of the water of the same breeding place shows most remarkable variations at different periods of the day It would be a mistake to speak of 'oxygen toleration limits' of these species, when it is found that the oxygen content of the same water may rise from a low to a very high value and fall again to its low level during the course of the day and yet the larvæ continue to thrive in the water all the time The records of several ponds investigated in this connection show that these diurnal variations are natural phenomena In this series of observations, estimations of the oxygen content of the water were carried out several times during the course of the day and night and the observations were continued for several days at a stretch The original observations have been confirmed by observations during each of the subsequent days

The rise and fall of the dissolved oxygen content of water in Pond No. RY at Sonarpur is represented in Chart 14 The period covered by this series is 12th to 14th November, 1925 On the 13th November, the oxygen content was as low as

2.15 mg per litre at 6 a.m. and it rose to 3.85 mg per litre at 3 p.m. on the same day. The same phenomenon is repeated on the following day, the 14th November. These diurnal variations are seen even more conspicuously in Pond No. BT, the observations on which were carried over the same period, 12th to 14th November (Chart 15). The variations in the latter pond are even more marked than in Pond RY. At 6 a.m. on the 13th, the oxygen content was at 2.95 mg per litre and at 3 p.m. on the same day, it had risen to 5.6 mg, nearly double what it was nine hours previously. A similar occurrence has been recorded on the following day, the 14th November.

CHART 14

*Diurnal fluctuation of dissolved oxygen—Pond RY*

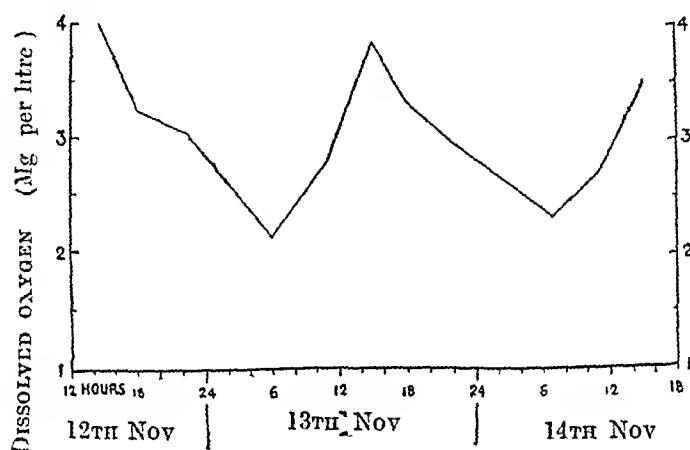
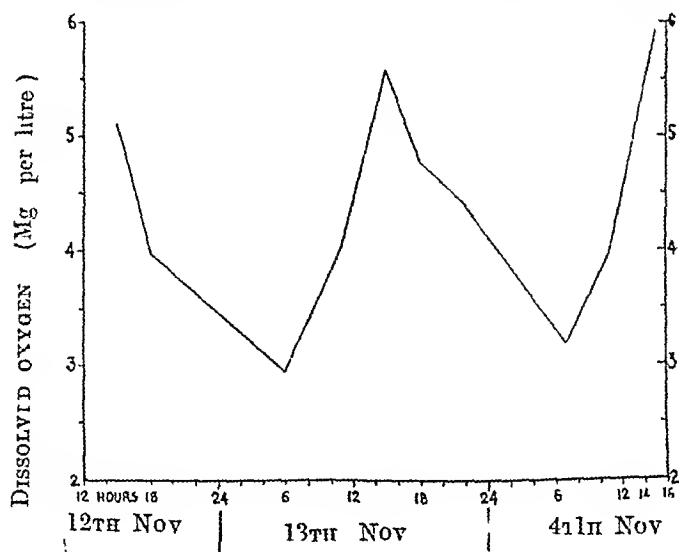


CHART 15

*Diurnal fluctuation of dissolved oxygen—Pond BT*

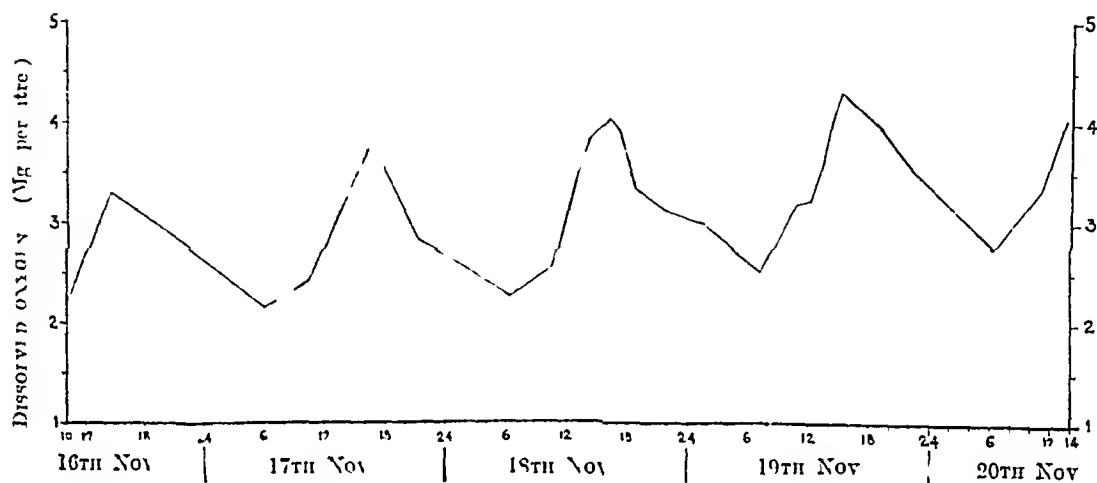


These observations were repeated again a few days later and on this occasion, the estimations were done at shorter intervals during the day and night and the

observations were continued for five days at a stretch. For two days, hourly estimations of the oxygen content of the water were carried out during day-time. The results of these estimations are given in Appendix III and the variation is

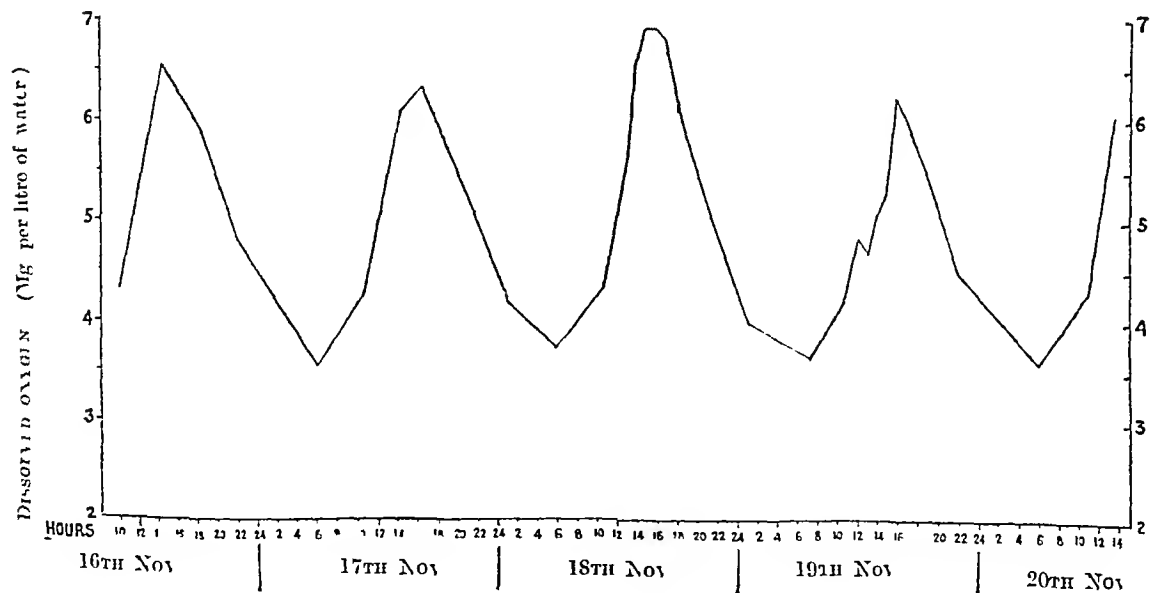
### CHART 16

#### *Diurnal variations of dissolved oxygen—Pond RY*



### CHART 17

#### *Diurnal variations of dissolved oxygen—Pond BT*



graphically represented in Charts 16 and 17. The period of observation was 16th to 20th November and the two ponds RY and BT were investigated. During each of the days, it is seen that the dissolved oxygen content is lowest at about 6 a.m. and reaches its highest level between 3 and 4 p.m. in the afternoon and thereafter declines gradually till it reaches its lowest level at 6 a.m. on the following day.

These records are of interest from the biological point of view. The fluctuations of the oxygen content of pond water are due to two factors, (1) the production of oxygen as a result of photosynthesis by the flora of the pond, and (2) the absorption of oxygen as a result of respiration by the plant life and animal life of the pond. While the former process is restricted to the period of sunshine, the latter goes on throughout the day and night. The important elements of plant life responsible for the enormous output of oxygen during day time are algæ, especially the very minute forms belonging to the families *Volvacaceæ* and *Pleurococcaceæ*. The actual output of oxygen by these algæ being directly dependent on the amount of sunshine, the maximum oxygen content of the water is reached between 2-30 and 4 p.m. in the afternoon. This is about the time when the temperature of the water is also at the highest. In this connection, it may be pointed out that the maximum temperature of land surface cut in the sun is reached at about 1-45 p.m.

It is interesting to note that the period of highest temperature is nearly the period of maximum dissolved oxygen content since both these factors are resultant of the amount of sunshine. This is mentioned here because the writer was warned to allow for the temperature of the water in estimating the amount of oxygen, since the amount of dissolved gases varies inversely with the temperature. But it has been found in the stagnant water ponds of Sonaipur that the temperature of the water rises simultaneously with the rise of dissolved oxygen concentration as the result of sunshine.

As was stated above, the processes of production of oxygen and absorption of oxygen go on side by side in the pond. During the night, the production of oxygen in the water is totally suspended and the amount of oxygen in the water gradually diminishes as the result of respiration by the plant and animal life of the pond. This process of using up of oxygen goes on till sunrise on the day following when the production of oxygen starts and checks the downward fall of the curve. Soon after this stage is reached, there is more production of oxygen than is used up by respiration and the oxygen curve rises gradually till it reaches its maximum for the day between 3 and 4 in the afternoon. With a lessening of the sunshine, the onward rise of the curve is stopped and the curve declines thereafter.

Plant physiologists demonstrate the existence side by side, of the two processes respiration and photosynthesis in plants exposed to sunlight. In plant tissues carrying on vigorous photosynthesis, the process of respiration is not ordinarily noticed, because it is masked by the more vigorous process of oxygen production. Even during the time when active photosynthesis is going on, the process of respiration could be demonstrated by shading the plant and thereby reducing or totally stopping photosynthetic activity, when the process of respiration which has been

going on all the time, becomes apparent. These same phenomena are demonstrable in a pond as well. It is possible for the oxygen figure to show a fall even at midday if the sun should be covered by a passing cloud. Such an event happened between 11 a.m. and 12 noon on the 19th November and this is reflected in the oxygen curves of the two ponds BT and RY (Charts 16 and 17). The oxygen curve which had been rising steadily, suddenly showed a decline, but when the effect of the cloud was over, the curve rose again soon after.

These results are of considerable biological interest and the writer has therefore diverged somewhat from the main question of the relation of dissolved oxygen to *Anopheles* breeding. The writer is not aware of any previous work on these very remarkable diurnal variations in the oxygen content of pond water.

In studying the relation of *Anopheles* breeding to the amount of dissolved oxygen, it was stated that the collection of samples was restricted to the morning hours between 8 and 10 a.m. In view of these diurnal variations, the importance of this precaution is apparent.

To revert again to the subject of dissolved oxygen in relation to *Anopheles* breeding at Sonarpur, the writer believes that in the light of the above researches on the enormous diurnal variations in the concentration of dissolved oxygen, there is no likelihood of obtaining any definite oxygen toleration limits for these species of *Anopheles*. If, on the other hand, we should study the aquatic vegetation and its relation to anopheles breeding, we should probably obtain less varying results. For instance, it has been found that the presence of a dense covering of the water surface by *Pistia* is usually associated with the presence of the two species *A. hyrcanus* and *pseudojamesi*. When the *Pistia* occurs sparsely, other species breed in addition to these, like *varuna* and *fuliginosus*. In the presence of *Eichhornia* in the water, we commonly find *A. varuna* alone by itself or in company with *hyrcanus* and *pseudojamesi*. When a pond is clear or has grass at edge, *A. varuna* is the most prolific breeder. Small ditches in the shade usually breed *A. barbuosus* and *A. varuna*. Where there is much decaying vegetation with rotting bamboo leaves and the pool is shaded, *A. barbuosus* is very common. Where sub-aquatic bushes of aquatic plants reach the water surface, *A. fuliginosus* is very common.

A more representative series of observations with a view to study the relation between the aquatic vegetation and the species of *Anopheles* is being carried out at Sonarpur and it is hoped that some more definite information will be had which would enable us to understand the variations in the distribution of the species in the different ponds and ditches.

The writer desires to acknowledge his indebtedness to Mr Narendranath Chatterjee for having carried out a series of tests for nitrites.

#### SUMMARY

Investigations on the dissolved oxygen concentration of water in stagnant ditches and ponds near Sonarpur were carried out with a view to study the relation of oxygen concentration to *Anopheles* breeding. The dissolved oxygen content



of ditches and ponds ranged from zero to 6.5 milligrams per litre of water, but the greater proportion of the breeding places had a dissolved oxygen content of 1 to 4 milligrams per litre

The six species of *Anopheles* investigated in this connection did not show any definite relation to the concentration of dissolved oxygen except in the case of *Anopheles pseudojamesi* and *hyrcanus* which showed a diminished prevalence with increasing oxygen concentration. But it is possible that even these two instances are mere coincidences and that this apparent relation is actually due to the association of these two species with the aquatic plant *Pistia*.

All the species of *Anopheles* studied were found to breed in all grades of dissolved oxygen concentration and there is no evidence of any 'oxygen toleration limits' in any of the species studied. It has been found that the surface water of ponds show remarkable variations in the concentration of dissolved oxygen at different periods of the day, and *Anopheles* larvae breeding in the pond are not affected in any way by the great fluctuation in the oxygen content of the water during the course of the day which seems to be a natural phenomenon.

The remarkable diurnal variations in the dissolved oxygen concentration in water of ponds has been studied in full detail. The oxygen concentration reaches the lowest level for the day at 6 a.m. and reaches the highest level between 2-30 and 3-30 p.m. These fluctuations are natural phenomena observed invariably during each of the days during which these observations were carried out and in every pond investigated. They are caused by the process of oxygen production through photosynthesis during day-time and the using up of oxygen through respiration by the plant and animal life of the pond throughout the day and night. During day-time, both the two processes go on side by side, but the process of oxygen production through photosynthesis is the more active process and thus masks the process of respiration. The highest oxygen concentration of the pond water is reached at a time when sunshine is greatest and when the temperature of the water is also at its highest. Minute algae play a very large part in the increase of the dissolved oxygen concentration of the water.

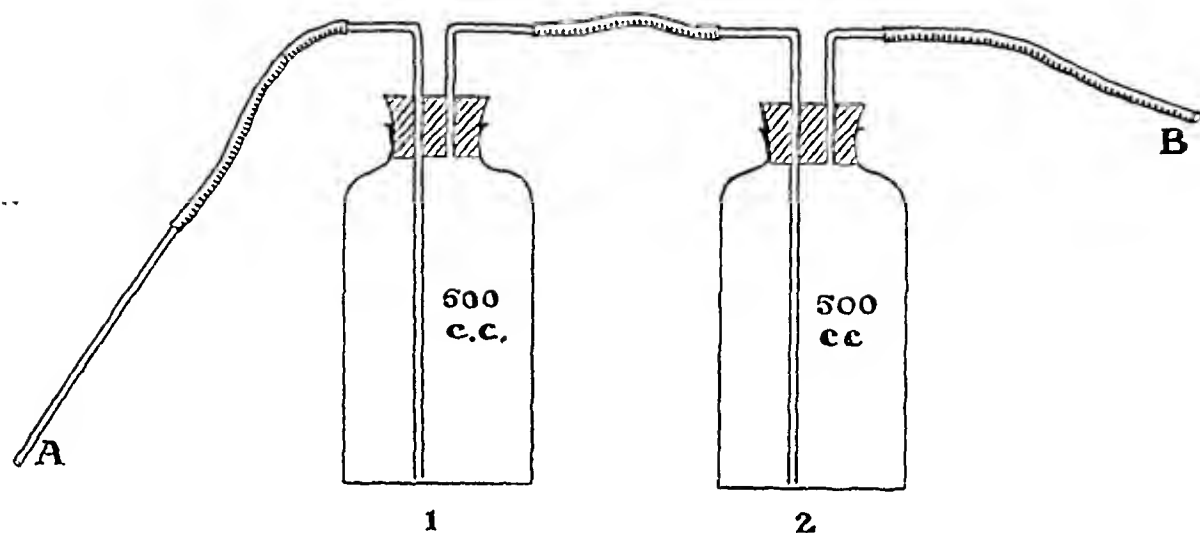
## APPENDIX I

### *Technique of water collection and estimation of dissolved oxygen*

The collection of water samples for dissolved oxygen estimation was done between 8 and 10 a.m.

A collecting apparatus as shown in the Text-figure was used for collecting water for oxygen estimation. The purpose of this apparatus is to obtain a sample of water which has had no contact with atmospheric oxygen during the process of collection. The apparatus consists of two bottles of about 500 c.c. capacity fitted with rubber stoppers and glass tubing as shown in figure. The inlet tube of each bottle reaches the bottom and the outlet tube is on a level with the stopper. All the joints are

tested for being air-tight. The end of tube A is placed in water about two feet away from the edge of the pond and at a depth of 1½ to 2 inches below the surface and air is sucked in at B. The first bottle fills up first and the second bottle gets filled in with the water from the first bottle and the latter gets a fresh sample of water which has had no contact with atmospheric oxygen during the process of collection.



FIGURE

When the second bottle is nearly full the collecting apparatus is taken out and tube A inserted into a dry reagent bottle of about 250 c.c. capacity and the water is slowly allowed to fill it by gravitation. When the bottle is full, a certain amount of water is allowed to overflow, the tube is then removed and the bottle stoppered immediately.

The estimation of dissolved oxygen of the sample is carried out as soon as possible after collection. The stopper of the reagent bottle containing the water sample is removed carefully and 1 c.c. of a saturated aqueous solution of manganous chloride is run in and this is followed by running in 1 c.c. of alkaline potassium iodide solution, the tip of the pipettes being placed below the surface of water so as to avoid agitation. The bottle is then stoppered and slowly rotated to ensure thorough mixing. The bottle is then placed in a dark cupboard for 15 minutes to allow the sediment to settle. When the sediment has completely settled, 3 c.c. of strong hydrochloric acid is added, the bottle is stoppered and slowly rotated to dissolve the precipitate. This process liberates an amount of iodine equivalent to the amount of the free oxygen which was present in the water. The contents of the bottle are then poured into a titrating flask and the amount of free iodine estimated by titration with thiosulphate solution. The titration is carried out as rapidly as

possible, starch solution being added towards the close of the titration. Since the strength of the thiosulphite solution varied from day to day, it was standardized daily against a solution of potassium permanganate of known strength.

The calculation of dissolved oxygen present in the water is done as follows.

Suppose that the exact capacity of the bottle is 252 c c. The quantity of the reagents added first, namely 2 c c, should be deducted from the capacity of the bottle. Supposing that 17.6 c c of thiosulphite solution was required for the titration and that 36.0 c c was found to be equivalent to 2 mg of oxygen by titrating it against standardized potassium permanganate solution, the following calculation would give the amount of dissolved oxygen in the water—

Capacity of bottle—252 c c, less 2 c c (amount of reagents added) = 250 c c

250 c c of water required 17.6 c c thiosulphite solution, which is equivalent to  $\frac{2 \times 17.6}{36}$  mg of oxygen

Dissolved oxygen in 1,000 c c of the same water would be equivalent to  $\frac{2 \times 17.6 \times 1000}{250 \times 36}$  mg of oxygen

The oxygen content of the water would thus be 3.95 mg per litre of water.

## APPENDIX II

### *A Frequency of occurrence of anopheles in ponds with different concentrations of dissolved oxygen*

	OXYGEN PER LITRE OF WATER						
	0—1 mg	1—2 mg	2—3 mg	3—4 mg	4—5 mg	5—6 mg	6—7 mg
<i>A. barbirostris</i>	$\frac{19}{34}$ 50 per cent	$\frac{61}{101}$ 62.4 per cent	$\frac{42}{71}$ 59.1 per cent	$\frac{41}{61}$ 70.5 per cent	$\frac{29}{41}$ 42.5 per cent	$\frac{16}{16}$ 60.0 per cent	$\frac{2}{2}$ 50.0 per cent
<i>A. hyrcanus</i>	$\frac{18}{38}$ 47.4 per cent	$\frac{40}{101}$ 48.5 per cent	$\frac{50}{71}$ 42.2 per cent	$\frac{20}{61}$ 32.8 per cent	$\frac{9}{47}$ 19.2 per cent	$\frac{10}{30}$ 30.0 per cent	$\frac{1}{4}$ 75.0 per cent
<i>A. fuliginosus</i>	$\frac{18}{68}$ 26 per cent	$\frac{10}{101}$ 40 per cent	$\frac{9}{71}$ 8.4 per cent	$\frac{10}{61}$ 16.4 per cent	$\frac{17}{47}$ 10.6 per cent	$\frac{10}{20}$ 20.0 per cent	$\frac{9}{9}$ 0.0 per cent

APPENDIX II—*conold*

	OXYGEN PER LITRE OF WATER						
	0—1 mg	1—2 mg	2—3 mg	3—4 mg	4—5 mg	5—6 mg	6—7 mg
<i>A. taruna</i>	63.3 per cent	78.2 per cent	77.4 per cent	77.0 per cent	51.6 per cent	70.0 per cent	50.0 per cent
<i>A. subpictus</i>	5.0 per cent	1.0 per cent	14.1 per cent	13.1 per cent	2.1 per cent	0.0 per cent	25.0 per cent
<i>A. pseudojamesi</i>	34.2 per cent	22.7 per cent	12.6 per cent	3.3 per cent	0.0 per cent	10.0 per cent	25.0 per cent

B *Average number of larvae of each species per breeding place in ponds with different concentrations of dissolved oxygen*

	OXYGEN PER LITRE OF WATER						
	0—1 mg	1—2 mg	2—3 mg	3—4 mg	4—5 mg	5—6 mg	6—7 mg
Total number of observations of each grade	38	101	71	61	47	10	4
<i>A. barbirostris</i>	5.1	3.9	4.9	5.0	2.4	2.6	12.5
<i>A. hyrcanus</i>	4.0	4.2	1.9	2.4	0.6	2.2	2.3
<i>A. fuliginosus</i>	0.3	0.8	1.4	1.4	1.0	2.9	0.0
<i>A. taruna</i>	8.2	9.3	9.4	10.1	1.4	7.6	5.8
<i>A. subpictus</i>	1.8	0.6	1.2	0.7	4.2	0.0	0.3
<i>A. pseudojamesi</i>	1.8	1.3	0.8	0.7	0.0	0.5	0.5

## APPENDIX III

*A Pond RY—Record of variation of dissolved oxygen content*

Date	12th November			13th November					14th November		
Time of collection	2 30 p m	6 0 p m	10 15 p m	6 0 a m	11 0 a m	3 0 p m	6-0 p m	10 0 p m	7 0 a m	10 45 a m	2 45 p m
Temperature of water, in degrees Centigrade	30	30	28	27	29	30	29	28	27	28	29
Dissolved oxygen in milligrams per litre	4 00	3 24	3 05	2 15	2 83	3 88	3 33	2 98	2 33	2 72	3 51

*B Pond BT—Record of variation of dissolved oxygen content*

Date	12th November			13th November				14th November		
Time of collection	2 45 p m	6 0 p m	6 0 a m	11 0 a m	3 0 p m	6 0 p m	10 0 p m	7 0 a m	10 45 a m	2 45 p m
Temperature of water, in degrees Centigrade	29	29	26	27	29	28	28	26	27	28
Dissolved oxygen in milli grams per litre	5 11	3 97	2 95	4 06	5 59	4 78	4 41	3 17	3 99	5 93

# APPENDIX III—*contd*

C Pond RY—Record of variation of oxygen content, (period 16th to 20th November)

Date	16th November				17th November				19th November									
	10.0 a.m.	2.30 p.m.	6.30 p.m.	10.0 p.m.	6.0 a.m.	10.30 a.m.	2.30 p.m.	4.30 p.m.	8.45 p.m.	12.45 p.m.	4.15 p.m.	10.30 a.m.	1.0 p.m.	2.0 p.m.	4.0 p.m.	5.0 p.m.	6.30 p.m.	9.30 p.m.
Time of collection																		
Temperature of water, in degrees Centigrade	27	29	26	26	25	27	27.5	27	26	25	25	27	28	28	27.5	27	26	25.5
Dissolved oxygen in milligrams per litre	2.20	3.29	3.02	2.78	2.15	2.42	3.46	3.80	2.84	2.61	2.27	2.51	3.54	3.82	1.03	3.00	3.35	4.12

Date	19th November								20th November					
	1 15 a m	7 15 a m	10 40 a m	12 0 noon	1 0 p m	2 0 p m	3 0 p m	4 0 p m	5 0 p m	7 0 p m	10 0 p m	6 15 a m	11 0 a m	1 30 p m
Time of collection														
Temperature of water, in degrees Centigrade	23	25	27	27 5	27 5	27 5	27	27	26	25 5	24 5	24	26	27 5
Dissolved oxygen in milligrams per litre	2 99	2 52	3 17	3 21	3 52	4 00	4 30	4 20	4 11	3 94	3 53	2 76	3 35	4 04

# APPENDIX III—*concd*

D Pond BT—Record of variation of oxygen content, (period 16th to 20th November)

Date.	16th November				17th November				18th November										
	10 0 a m	2 30 p m	6 30 p m	10 0 p m	6 0 a m	10 30 a m	2 30 p m	4 30 p m	8 45 p m	12 45 a m	6 15 a m	10 30 a m	1-0 p m	2 0 p m	3 0 p m	4 0 p m	5 0 p m	6 30 p m	9-30 p m
Time of collection																			
Temperature of water, in degrees Centigrade	26	28	27	25	24 5	27	28	27	25 5	24 5	25	26 5	28 5	29	29	28	27	26	25
Dissolved oxygen in milligrams per litre	4 33	6 54	5 91	4 82	3 57	4 28	6 10	6 34	5 22	4 17	3 75	4 33	5 04	6 55	6 94	6 93	6 84	5 99	5 05

Date	19th November										20th November			
	1 15 a m	7 15 a m	10 30 a m	12 0 noon	1 0 p m	2 0 p m	3 0 p m	4 0 p m	5 0 p m	7 0 p m	10 0 p m	6 0 a m	11 0 a m	1 30 p m
Time of collection														
Temperature of water, in degrees Centigrade	24 5	24	26 5	26 8	27 5	28	27	26	26	25	24 5	23	26	28
Dissolved oxygen in milligrams per litre	4 00	3 65	4 20	4 84	4 70	5 07	5 30	6 22	6 02	5 45	4 49	3 53	4 32	6 06

# THE LARVA OF *ANOPHELES TURKHUDI*

BY

M O T IYENGAR, B.A., F.Z.S.,

Entomologist, Bengal Public Health Department, Calcutta School of  
Tropical Medicine

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THE larval stage of *Anopheles turkhudi* Easton has been imperfectly known and instances are not uncommon of other species of *Anopheles* being mistakenly identified as *A. turkhudi*. It is desirable therefore that a complete description of the larva is published. The larva of *A. turkhudi* has several very characteristic features which when known would probably eliminate such mistaken identifications.

The egg of *A. turkhudi* has been shown by Stephens and Christophers (1902) to be very characteristic, their figures show that these eggs are quite unlike those of any other species of *Anopheles*. They had also described the clypeal and palmate hairs of the larva. The present description of the larva brings out its several very characteristic features, some of which are probably unique.

At once the most characteristic feature of the larva of *A. turkhudi* is the shape of the mouth-brushes as seen in an exerted condition. These brushes project straight out on either side of the head and are quite unlike those of other anopheline larvæ. The characteristic mouth-brushes of *A. turkhudi* are seen in Plate LXXIII, figs 1 & 2, and they are strikingly different from the ordinary type of mouth-brush of *Anopheles* larvæ seen in Plate LXXIII, fig 3. Another feature of the larva of *A. turkhudi* is the primitive condition of the frontal hairs which are different from other anophelines in that they are poorly developed and sparsely branched. In larvæ of other species of *Anopheles* the frontal hairs are conspicuous, densely branched and plumose. The only exceptions known are the tree-hole breeding anophelines (Iyengar, 1930), in which the frontal hairs are thin, short and inconspicuous. Other characteristics of *A. turkhudi* larva are the entirely unbranched clypeal hairs the very long posterior clypeal hairs, minute and poorly developed palmate hairs which are present only on the three abdominal segments, 4th, 5th and 6th. The resting attitude of this larva has been noted by previous workers to be different from the ordinary *Anopheles* larva, in that the larva rests at an angle from the water surface instead of being parallel to it.



The following is a detailed description of the larva of *Anopheles turkhudi*

General appearance and coloration very similar to *A. subpictus* larva Length 5 to 6 mm , breadth of thorax 1.5 mm

*Head* Mouth-brushes very characteristic in appearance when exerted (Plate LXXIII, figs 1 & 2) They stand out straight on either side of the clypeus This characteristic appearance cannot be made out when the feeding brushes are withdrawn underneath the anterior part of the clypeus The bristles of the brushes are stiff and straight and feebly hooked at the tip The exerted mouth-brushes of *A. turkhudi* resemble somewhat the mouth-brushes of a *Megasthinus* larva No other species of *Anopheles* has been observed to have a mouth-brush as in *A. turkhudi*, the ordinary type being that seen in Plate LXXIII, fig 3

Clypeal hairs are all simple, unbranched and unfrayed The inner hairs are long, the outer ones are thin and about half the length of the inner clypeal hairs, and start slightly behind the base of the latter Posterior clypeal hairs are very long and they stand out erect They are as thick and as long as the inner clypeal hairs and project beyond the profile of the clypeus They start far apart, on a level with the bases of the external clypeal hairs or even external to these The disposition of the clypeal hairs is seen in Text-figures c and d The bases of the three pairs of clypeal hairs as seen in a flattened clypeus are shown in Text-figure e

Frontal hairs are poorly developed and differ from those of most other known species of *Anopheles* The hairs are quite thick but are lacking in the plumose branching present in other species The inner frontal hairs are long and thick, and have 2 to 3 branches The median frontal hairs have 2-3-4 branches and the external hairs have 3-4 branches

Antenna devoid of any large branched hair it has a small unbranched hair at about half-way up the shaft, spines on shaft short and minute Terminal branched hair of antenna, normal in character Basal hair of antenna plumose and normal

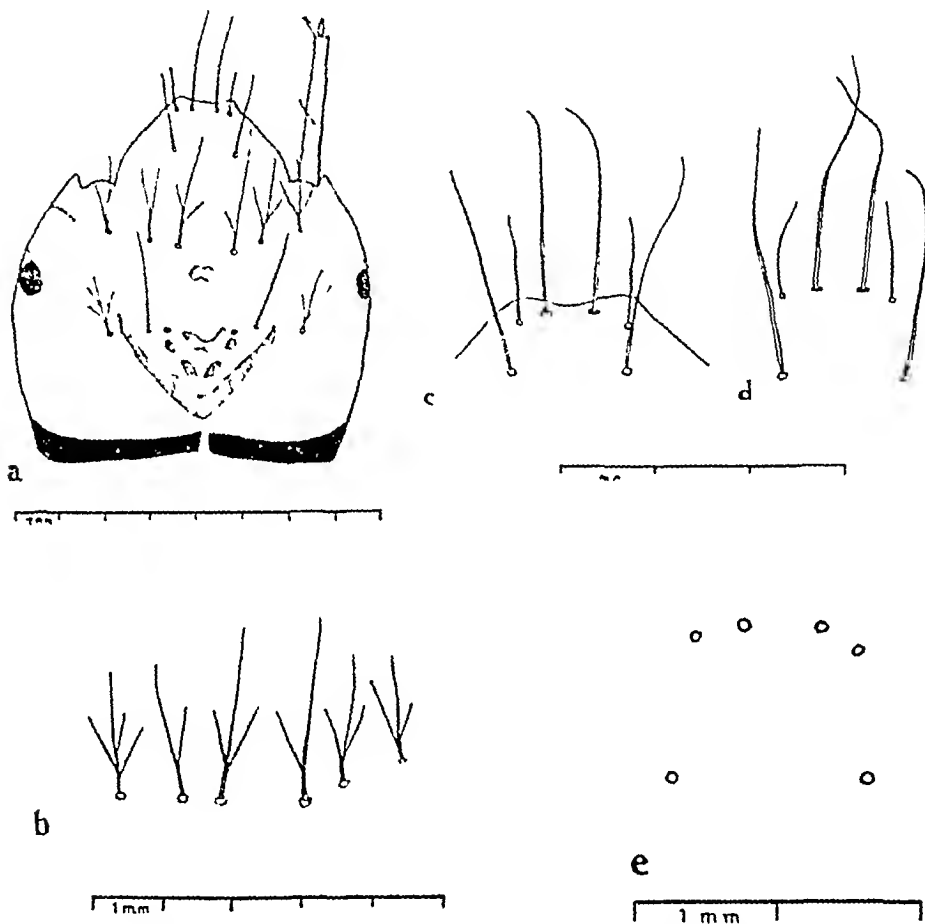
*Occipital hairs* Inner ones are long and simple, external ones 3 to 5 branched all the occipital hairs are inserted in a line Head pattern (Text-figure a) not conspicuous and similar to that of *A. subpictus*

*Thorax* Shoulder-hairs are not conspicuously pigmented The inner and median shoulder-hairs are plumosely branched and possess well developed roots They arise far apart from each other The inner shoulder-hair has about 18 to 22 branches, and the median one has 16 to 21 branches The external shoulder-hair is simple as normally, but it is uniquely very long and thick, often nearly as long as the median shoulder-hair In most other species of *Anopheles* the external shoulder-hair is small and minute

Palmate hairs absent on thorax and on the first three abdominal segments

*Abdomen* Large branched lateral hairs of abdomen, normal, i.e., two on each side segments 1 and 2, and one on either side on segment 3 Palmate hairs present on segments 3 to 6 only They are absent on segment 7 The number of leaflets of the palmate hairs is small, usually about 9 to 12 The leaflets are short,

measuring about  $11\ \mu$  long, and 5 to  $8\ \mu$  broad. At about three-fourths its length, the leaflet constricts abruptly and tapers to the tip. The tip is usually truncate but may occasionally be acute. There is no typical filament, unless the terminal portion of the leaflet is termed as such. There is no great difference in



Text figure *Inopheles turkhuḍi* larva (camera lucida drawings)

- a Head
- b Frontal hairs
- c and d Clypeal hairs
- e Bases of clypeal hairs as seen in a clypeus dissected and flattened out

appearance of the leaflets in the three pairs of palmate hairs that occur on the larva. The leaflets are usually brown in colour.

Scutæ are present but they are not conspicuous or very large.

The characteristic features of *A. turkhuḍi* larva are—(1) distinctive mouth-brushes, (2) simple clypeal hairs, (3) long posterior clypeal hairs, (4) sparsely

branched frontal hairs, (5) the shoulder-hairs, (6) minute palmate hairs restricted to segments 3 to 6 of abdomen

The egg and the important features of the larva of *A. turkhudi* have been described by Stephens and Christophers (1902) and later by James and Liston (1912). The latter authors have correctly figured the peculiar mouth-brushes of this species.

Patton (1905) in describing the larva of *A. azniki*, which has since been placed as a variety of *A. turkhudi* by Christophers, had observed that *A. azniki* has a mouth-brush similar to the ones described above in *A. turkhudi*. He remarked that the feeding brushes of the larva of *azniki* are placed laterally. Patton observed that the larva suspends itself in the water like a culex larva and that its body below the water surface from the head up to the 2nd segment are submerged (Patton, 1905, p. 633). It is evident that this form from Aden is very closely allied to *A. turkhudi*. A further examination of its larva would determine whether it is identical with *A. turkhudi* or is a distinct variety.

The larvæ of species allied to *A. turkhudi* are not known sufficiently well. It is desirable that the larvæ of *var. azniki* Patton, *var. persicus* Edw., *A. hispaniola* Theob., *A. italicus* Raff., *A. flaviceps* Edw., and *A. multicolor* Camb. are studied in order to ascertain their relationship to *A. turkhudi*.

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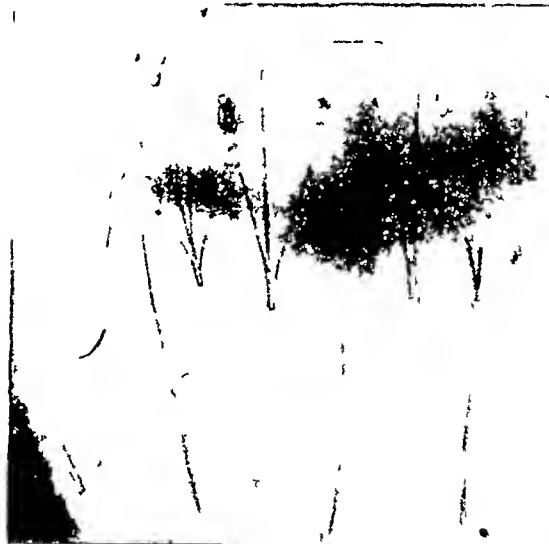
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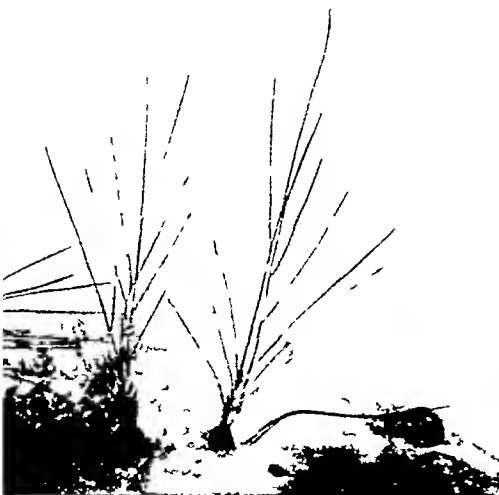
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4



5



6



### EXPLANATION OF PLATE LXXIII

- Figs. 1 and 2 Head of *Anopheles turlhudi* larva showing characteristic mouth-brushes  
 Fig. 3 Head of *Anopheles majidi* larva showing the normal type of mouth-brush  
           of anopheline larva  
 „ 4 *A. turlhudi* larva showing frontal hairs (above) and occipital hairs  
 „ 5 Shoulder-hairs of *A. turlhudi* larva  
 „ 6 Clypeal hairs of same  
 Figs. 7 to 11 Palmate hairs (segments 1 and 5)  
           Fig. 7 is more magnified than Figs. 8 to 11  
 Fig. 12 Scale for Figs. 8 to 11, each division is equal to 10  $\mu$



# A 'STANDARD ANTIGEN' FOR USE IN ALL FLOCCULATION TESTS FOR SYPHILIS

BY

K V KRISHNAN, M B M R C P I , D B (London)

(Kala-azar Commission )

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THE great simplicity and fair reliability of the different flocculation tests for syphilis are making them increasingly popular all over the world. In the United States the Kahn test has practically replaced the Wassermann reaction in most of the important laboratories. In Great Britain, the staunch adherence to the time honoured Wassermann reaction is being slowly broken down and it is now not uncommon to see some of the leading laboratories in London, Glasgow and Edinburgh adopting the flocculation test with very promising results. In continental laboratories also, one or other of the many flocculation tests is being used in the routine diagnosis of syphilis. There is no doubt that with certain improvements in technique and in the method of standardization of the antigens used the results obtained with these tests will be as reliable and as specific as those of the Wassermann reaction itself. For, after all, when one considers the relation between the flocculation tests and the Wassermann reaction, it appears the interaction between the serum and antigen is essentially the same in the two cases. In the flocculation tests the ratio between the reagents is such that the end result of their interaction becomes readily visible, whereas in the Wassermann reaction the ratio is quite different and the end result is not visible, a special indicator in the nature of a hæmolytic system being used to detect the reaction. Dean, who is a recognized authority on the nature of these reactions writes 'the process which causes precipitation and complement fixation is in all probability the aggregation of molecules present in the antisera by the action of the antigen. When sufficient antigen is present aggregation takes place energetically and flocculi are rapidly separated. These are the conditions favourable to the precipitation reaction. If a relatively smaller amount of antigen is present the process of aggregation is slower and less complete. These are the conditions where the individual particles which form the precipitate are extremely small, but the surface afforded by all the particles of such a precipitate must be larger than that offered by a precipitate composed of large flocculi. It is indeed possible that there is a direct relationship between the surface area of the particles of the precipitate and the amount of complement adsorbed



It can, in fact, be shown experimentally that either complement fixation or precipitation can be produced with the same antigen and the same antibody by merely varying the proportions of the mixtures' From this work of Dean, which has been further confirmed by Sachs, it is but just to presume that in the very near future these simple flocculation tests will become the standard methods for the routine diagnosis of syphilis

Although a very large number of flocculation tests have been described for the diagnosis of syphilis, the two most popular are the Sachs Georgi and the Kahn tests Everyone who is familiar with these tests will certainly agree that neither of them have yet been perfected enough completely to replace the Wassermann reaction While both of them have numerous advantages over the other flocculation tests, neither of them has shown in the past absolute agreement with the Wassermann reaction in all hands This is all the more surprising when one remembers that the Wassermann reaction is the more complicated reaction of the two and necessitates the use of a larger number of reagents some of which are very difficult to standardize accurately However, a detailed study of the two flocculation tests above referred to does reveal why this lack of agreement with the Wassermann reaction exists

#### *The Sachs Georgi Test*

This is, of course, the simplest and the cheapest of the flocculation tests

The antigen is a cholesterolized alcoholic extract of wet heart muscle and is easily made

The articles of apparatus required to perform the test are the ordinary graduated pipettes (1 to 10 c c), glass tubes (size 7.5 cm length  $\times$  1 cm diameter) and incubator All these are usually available in every laboratory

The test itself is done by adding one part of a 1 in 6 dilution of the antigen to two parts of varying dilutions (1 in 2, 1 in 4, 1 in 8, 1 in 16, 1 in 32 and 1 in 64) of freshly inactivated serum and incubating the mixture from 4 to 12 hours at 37°C, before reading off the results The quantities of the reagents used are large (4 c c and 2 c c) and do not allow of any great error from measurement as in the Kahn test

The results obtained with the Sachs Georgi test in the past though fairly satisfactory, did not completely agree with those of the Wassermann reaction This lack of agreement was chiefly due to the absence of an efficient method of standardizing the antigen This defect, however, has recently been remedied by the writer who has described a method of standardization by which a 'standard antigen' of uniform optimum sensitiveness could easily be obtained The results given by this standard antigen in several hundreds of Sachs Georgi tests carried out in the Department of Bacteriology of Edinburgh University were in complete agreement with those of the Wassermann reaction This antigen is also being used for the Wassermann reaction in a higher dilution (1 in 12) with very satisfactory results (unpublished)

The second complaint against the Sachs Georgi test is that it is not as rapid as the Kahn, the results being obtained only at the end of 4 to 12 hours' incubation. This delay, if it may be called so, is inevitable due to the very dilute form in which both serum and antigen are used in the test. It is well known that in all precipitation reactions the time taken to obtain results is closely related to the ratio and the concentration of the reagents used. The nearer these two factors approach the optimum conditions the quicker the result. Also mechanical agitation hastens flocculation. Taking into consideration the fact that syphilis is not an acute illness in which a very urgent laboratory diagnosis is needed, a delay of 4 to 12 hours in obtaining results is not to be considered a point against the test.

The third and probably the gravest charge against the Sachs Georgi is that, as a result of incubation for about 12 hours bacterial growth is likely to occur in the serum antigen mixtures. This would if present, undoubtedly interfere with the reading of results. As no aseptic precautions are taken in the performance of the test this objection is certainly of some importance. Our experience with a large number of these tests shows that with ordinary cleanliness and care this objection becomes indeed very negligible.

### *The Kahn Test*

The greatest advantage of this test is the rapidity with which results can be obtained.

The antigen is made by a more elaborate method and involves greater time, labour and cost. It is a cholesterolized alcoholic extract of ether-extracted dry, powdered heart muscle.

The apparatus required to perform the test is not quite as simple as in the Sachs Georgi, special accurately graduated pipettes, shaking machines, etc. being required. These also help in enhancing the relative cost of the test.

The test itself is very simple and is performed by adding varying amounts (0.5 c.c., 0.25 c.c., 0.125 c.c.) of the antigen saline suspension (1+11 saline) to a definite quantity (0.15 c.c.) of freshly inactivated serum and shaking the mixture in an electric shaker for 3 minutes. The mechanical agitation, together with the optimum concentration of the two reagents used, help to bring about the maximum flocculation in the minimum period of time. To each of the tubes after the shaking 1 c.c. of saline is added and the results read off. The accurate measuring of the very minute quantities of the reagents used is at times difficult and proves a drawback to the test.

A study of the results of the Kahn test shows that in most hands, there is very close agreement with those of the Wassermann reaction. The lack of complete agreement sometimes complained of is mainly brought about either by errors in the measuring of the very minute quantities of the antigen saline suspension or by the use of antigens imperfectly standardized. The latter, probably, is more often the case, for in the experience of many, the Kahn antigen is rather difficult of accurate standardization even when one follows his directions fully. Some of

the antigens obtained from Kahn himself are at times found to be below the optimum sensitiveness

From the observations made above it is perhaps justifiable to state that the chief drawback in these flocculation tests is, probably, the absence of a reliable method for the efficient standardization of the antigen used. When once a suitable method is available, the antigen so prepared can be used not only with absolute reliability in all the different flocculation tests but also in the Wassermann reaction. The only difference will be in the dilutions of the antigen used, this, of course, varying with the different tests.

One of the objects in presenting this work has been to show how the 'standard antigen' recommended by the writer for use in Mackie's modification of the Sachs Georgi reaction can also be used in the Kahn test with more satisfactory results than Kahn antigen itself. Furthermore an attempt has been made to show how, by combining the use of this 'standard antigen' with some of the best features of the Kahn test, a flocculation test of great simplicity, economy and efficiency can be evolved. It is not at all the intention to add one more test to the already overcrowded list of flocculation reactions but rather to suggest a highly reliable and simple method suitable for Indian conditions. The fact that the 'standard antigen' could be used not only in the Sachs Georgi and Kahn flocculation tests but also in the Wassermann reaction with absolutely reliable results is evidence in favour of calling it the 'standard antigen'.

#### *The Standard Antigen*

In a previous article (Krishnan, 1929) a method of standardizing antigens for use in Mackie's modification of the Sachs Georgi reaction was described. This is a cholesterolized alcoholic solution of wet sheep heart muscle standardized to contain a total lipid-cholesterol content of 1.07 per cent and a cholesterol-content less than 25 per cent. Fair quantities of a number of different antigens prepared and standardized by the above method was available for further study. It was thought worth while to see if these 'standard antigens' could be used in place of the Kahn antigen in his test. Theoretically at least this should be possible if our method of standardization was reliable. From previous studies it was evident that the 'standard antigen' contained about half the concentration of lipoids and cholesterol that was in the Kahn antigen. Therefore it was decided to use double the quantity of the antigen saline suspension recommended by Kahn. Thus to three tubes containing each 15 c.c. of freshly inactivated serum, 1, 0.5 and 0.25 c.c. of a 1 in 2 antigen saline suspension was added and the tubes shaken with the hand (no electric shaker being available) for 5 minutes, at the end of which, 1 c.c. of saline was added to each tube and the result read off. Side by side, using the same sera, the Kahn test with Kahn's own standard antigen (Lot No. 17) and the technique recommended by him, was also done. The only difference was that, instead of using the electric shaker for 3 minutes, the tubes were shaken by the hand for 5 minutes. The results of 62 sera containing known positives and negatives are given in Tables *IA*, *IB* and *IC*.

TABLE IA

Giving results of strongly positive sera

Number of Sera	* KAHN ANTIGEN			STANDARD I			STANDARD II			STANDARD V			STANDARD VI		
	05	025	0125	1	05	025	1	05	025	1	05	025	1	05	025
1	2	2	3		3	3	2	3	3	2	2+				
2	3+	3+	3	3	3+	3+	3	3+	3+	3	3+	3+			
3	3	3	3	3	3+	3+	3	3	3+	3	3	3			
4	2	3	3	2	3+	3+	2	3	3+	2	3	3			
5	3	4	1	3	1	1	2+	1	1	2+	1	1			
6	4	1	1	1	1	1	4	1	1	1	1	1			
7	1	1	1	1	1	1	1	1	1	1	1	1			
8	3	3	4	3	3	1	3	3	1				3	3	1
9	3	2	3	3	2	2+	3	3	3+	3	2	2	3	3	3
10	3	1	3	3	3	3+	3	3	3+	3	3	3	3	3	3
11	3	3	3	3	3+	3+	3	3+	3	3	3	2+	3	3	2+
12	3	3	3	3	3+	3+	3	3+	3+	3	3	3	3	3	3

\*4 Signifies complete flocculation and clear supernatant fluid  
3 Signifies marked flocculation and slightly opalescent supernatant fluid  
2 Signifies moderate flocculation visible to naked eye, and opalescent supernatant fluid  
1 Signifies slight flocculation visible distinctly with hand lens  
+ Signifies flocculation less than 1  
+ Added to any number signifies flocculation slightly more than that number

TABLE IA—*concl'd*

Number of Sera	* KAHN ANTIGEN			STANDARD I			STANDARD II			STANDARD N			STANDARD V			STANDARD VI		
	06	025	0125	I	05	025	I	05	025	I	05	025	I	05	025	I	05	025
13	3	3	2	3	2	2+	3	3	2+				3	2	2	3	3	2
14	3	2	2	2	3	3	3	3	2+				3	2	2	3	2	2+
15	3	2	2	3	3	2	3	2+	2				3	2	2	3	2+	2+
16	3	3+	3	2+	3	3+	3	3	3				3	3	3	3	3+	3+
17	2	3	3	2	3+	3+	2	3	3+				2	2	3	2	3+	3
18	3+	3	2	3	3+	3	2+	3	3+				3	3	3	3	3+	3
19	3	3+	2+	3	3	3	2+	2+	3+				3	3	3	3	3	3+
20	2	2+	3	2	3	3	2	2	3				2	2+	3	2	3	3
21	3	3	3	2+	3+	3+	2	3	3+			3						
22	3	3	2+	3	3+	3	2+	3	3			3						
23	3	3+	2+	3	3+	3	2+	3	3			3						
24	3	3	3	2+	3	3	2+	3	3			3						
25	3	3	3	3	3+	3+	3	3	3+			3						

\* 4 Signifies complete flocculation and clear supernatant fluid

3 Signifies marked flocculation and slightly opalescent supernatant fluid

2 Signifies moderate flocculation visible to naked eye, and opalescent supernatant fluid

1 Signifies slight flocculation visible distinctly with hand lens

+ Signifies flocculation less than 1

+ Added to any number signifies flocculation slightly more than that number

TABLE IB  
Giving results of weakly positive sera

Number of Sera	Kahn Antigen			STANDARD I				STANDARD II				STANDARD N				STANDARD V				STANDARD VI			
	05	025	0125	1	05	1+	025	1	05	025	1	05	025	1	05	025	1	05	025	1	05	025	
1	-	1	1	-	1+	1+	1-	-	1	1	1	1	1	1	1	1	1	1	1	1	1	025	
2	-	1	+	-	1	1+	1+	-	1	1	1	1	1	1	1	1	1	1	1	1	1	025	
3	1	2	2	1	2+	2+	2+	1	2	2+	1	2	2	2	2	2	2	2	2	2	2	025	
4	+	1	2	+	1	1	2	-	1	2	-	1	2	-	1	2	-	1	2	-	1	2	
5	-	1	1	-	1	1+	1+	-	1	1+	-	1	1+	-	1	1+	-	1	1+	-	1	2	
6	2	2	1	2	2	1+	2	1+	2	2	2	2	2	2	2	2	2	2	2	2	2	1	
7	-	+	1	-	1	2	2	-	+	2	-	+	2	-	+	2	-	+	2	-	+	2	
8	-	+	1	-	+	1+	1	-	+	1	-	+	1	-	+	1	-	+	1	-	+	1	
9	-	+	1	-	+	1+	1+	-	+	1+	-	+	1+	-	+	1+	-	+	1+	-	+	1	
10	2	1	1	2+	1+	1	1	2+	1+	1	2+	1+	1	2+	1	2+	1	2+	1	2+	1	1	
11	2+	2	2	3	2+	2	2+	3	2+	2	2+	2	2+	2	2	2+	2	2	2	2	2	1	

TABLE IC

*Twenty-six known negative sera were also tested All agreed except two which gave results as follows —*

Number of Sera	KAHN ANTIGEN			STANDARD I			STANDARD II			STANDARD V		
	05	025	0125	1	05	025	1	05	025	1	05	025
1	—	—	1	—	—	—	—	—	—	—	—	—
2	—	—	1	—	—	—	—	—	—	—	—	—

For purposes of reporting these would be taken as negative to Kahn's test

It will be seen that there was complete agreement in the case of the strongly positive sera. With the weakly reacting sera, the 'standard antigen' gave better and clearer results than Kahn's antigen. With regard to the sera giving a negative Wassermann reaction Kahn's test showed in two cases, slight flocculation in the third tube, while the 'standard antigen' did not. These two sera, however, would have also been reported as negative to Kahn's test.

#### *Modifications in Technique*

As already pointed out one of the probable sources of error in the Kahn test is the use of very minute amounts of the antigen saline suspension. This is corrected to a certain extent by the use of the 'standard antigen' in place of Kahn's. It is commonly recognized that for the measurement of small quantities the dropping method using standardized pasteur pipettes is relatively more accurate than the volumetric method using graduated pipettes. Also the apparatus used in the former method is easily made and involves less cost. Hence it was decided to see if the dropping pipette method was really dependable for employment in the flocculation test above described with the 'standard antigen'.

In all precipitation tests it is the ratio between the reagents that is important and not the actual quantities used. The ratio of the serum to the 'standard antigen' dilution used in Kahn test was 6 : 4, 6 : 2, 6 : 1. Hence, maintaining the same ratio, tests were performed using the dropping pipette method. The pipettes for use were all standardized beforehand to ensure uniformity in the size of the drops. This was done by —

1. Having the lower ends of all pipettes of the same external diameter. Their ends were passed through a wire ring and, at the place where the pipettes were held tight by the ring, they were filed off.

2 Maintaining a uniform rate of delivery of drops—the faster they are delivered the larger the drops and the slower they are delivered the smaller the drops. The rate of about 1 drop a second was found satisfactory.

3 Having the temperatures of the reagents used the same as that of the room. As heat diminishes surface tension the hotter the fluid the smaller the drop. Hence the serum after being inactivated was brought down to the temperature of the room, before use.

4 Holding the pipettes while in use always vertical. If held obliquely the size of drop is bound to vary.

*The test*—This was done by adding to 3 tubes as in the Kahn test, 4, 2 and 1 drop respectively of a 1 in 1 antigen saline suspension and then adding to each tube 6 drops of undiluted, inactivated serum. The tubes were shaken with the hand for 5 minutes at the end of which 1 c.c. of saline was added to each and the result read off. Side by side the same sera were tested using the volumetric method for comparison. In all, nineteen sera containing known positives and negatives were examined. Table II gives the results obtained. The agreement was good and the dropping-pipette method was found to be as reliable as the volumetric method.

TABLE II

VOLUMETRIC METHOD				DROPPING PIPETTE METHOD		
Standard Antigen						
	1	05	025	1	05	025
1	3	3	3	3	3	3+
2	4	4	4	3	4	4
3	3	3+	3	3	3	3
4	2	3	3+	2	2+	3+
5	3	3	3	3	2+	3
6	3	3	3	3	3	3+
7	2	3	3	2	3	3
8	2	2	2	2	2	2
9	4	4	4	4	4	1

Ten negative sera were also tested with complete agreement

### CONCLUSIONS

1 An antigen, called the 'standard antigen,' which is easy to prepare and standardize, is recommended for the Kahn test.



main collecting tubules pass forwards and outwards to the sides of the body. Up to the level of the middle of the ventral sucker the ducts remain narrow. They then turn inwards and form a loop, converging towards each other, but remaining apart, after describing a semicircle they are again directed forwards. At this stage the tubes widen abruptly and take an almost straight course until they reach the margin of the pharynx, where they become narrow, and again assume the former dimensions. This dilated portion of the tube contains 6 to 8 spherical granules of excretory nature. The ducts now form a loop and pass backwards. The posteriorly directed tubules are hidden in the greater part of their length under the main collecting tubules and are visible only at places where they are not directly under them. Wherever they are visible they are seen to run internal to the main excretory tubules.

At about the level of the anterior end of the ventral sucker, where the first excretory granule is situated, the thin deflected excretory tubule on each side crosses the main excretory duct, and is continued back as a thin narrow tube running external to the main excretory duct, it branches into two capillaries, one takes an anterior course and the other goes towards the excretory vesicle. A pair of flame-cells is present at the extremity of the posterior branch. I could not trace any flame-cell connected with the anterior branch.

From the posterior margin of the excretory vesicle a thin duct is given off, which passes into the tail. It opens to the outside just before the tail tapers abruptly on the left side of the larva. There is no duct in the posterior smooth portion of the tail.

Excretory granules are usually oval or circular in outline, but sometimes when two or three are joined together the circular outline is lost (Plate LXXIV).

I have been able to detect only ten pairs of flame-cells. Six are pre-acetabular and four post-acetabular in position. At the external corner of the anterior loop a branch is received, which runs forwards and outwards and ends in a flame-cell on a level with the posterior margin of the oral sucker. In the region of the fourth excretory granule a small branch is received by the thin deflected excretory tubule, this branch is formed by the union of two capillaries. The posterior capillary ends in a flame-cell. I was unable to detect any flame-cell connected with the anterior capillary. A pair of flame-cells is present on the anterior surface of the acetabulum. They lie so close to the thin deflected excretory tubule that I could not detect any branch leading to them.

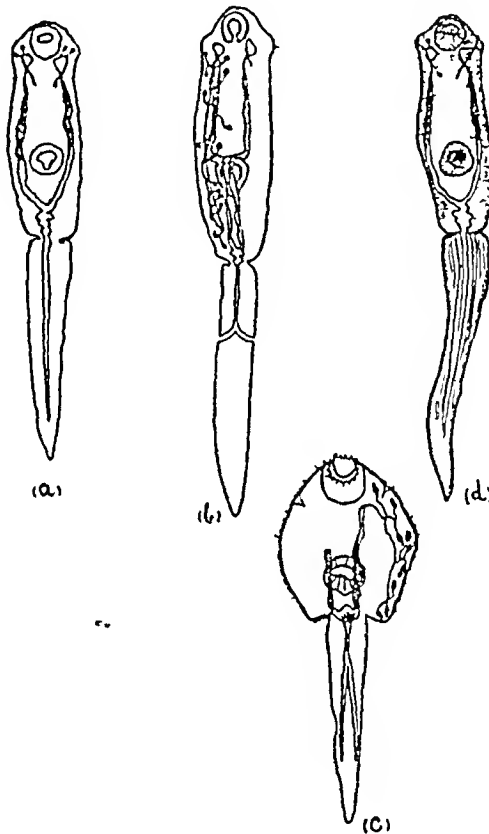
In the post-acetabular region of the body on either side, are given off from the excretory tubule a number of branches, but only four pairs of flame-cells were seen, three pairs on the outer side and one on the inner side of the tubule.

*Probable evolution of the excretory system —*

Faust (Excretory System in Digenea II. *Biological Bulletin*, No XXXVI, 5, 1919, p. 323) has stated that 'the manner in which the flame-cells' are

distributed, together with their number, and the course of the tubules in the tail offer a method for separating larval Echinostomes into two quite distinct groups '.

In the first and more simple group, a median excretory canal runs along the whole length of the tail, to open into the bladder posteriorly, and from the anterior



TEXT FIG 2

- (a) *Cercaria chisolenata*, Faust, ventral view, showing excretory system  
 (b) *Cercaria complexa* nov sp, ventral view, showing excretory system  
 (c) *Cercaria circumstricata*, dorsal view  
 (d) *Cercaria chisolenata*, dorsal view, showing excretory system

N B—The above text figures are exact tracings from the following journals —

Ref (a) 'The Excretory System in Digenea' by E C Faust, p 324, fig 1 *Biol Bull*, Vol XXXVI, No 5, 1919

(b) *Ibid*, p 324, fig 2

(c) 'Notes on Larval Flukes from China' by E C Faust *Parasitology*, Vol XIV, Nos 3 and 4, 1922, Plate XXII, fig 16

(d) 'Studies on Illinois Cercaria' by E C Faust *Parasitology*, Vol IV, pp 93—110, Plate I, fig 9, 1918

region of the bladder two collecting tubules run forward to the anterior end of the body, and in the region of the oral sucker form a loop or a triangular flexure in which three flame-cells arise—these being the only ones in the whole body. Examples of these forms are *C. chisolenata*, Faust, and *C. trisolenata*, Faust (see Text-fig 2a)

In the second more complex group the main excretory tube of the tail, after a short distance bifurcates and opens on the lateral margin, while in the body, the main collecting tube does not stop short at the triangular flexure, but is commonly continued to the posterior region of the body where it again bends forwards and runs to the region of the pharynx. Examples of this type are the cercaria of *Echinostomum revolutum* (Froelech) (vide Jhonson, 1920), and *Cercariae Indicae* XXXIII, Sewell, and *Cercariae Indicae* XII, Sewell.

Sewell has subdivided the Echinostomes into a number of subgroups, and according to him this second type of excretory system occurs in the 'Echinata' group, whereas in the 'Echinotoides' and 'Coronata' groups the ascending tubules bifurcate opposite the level of the acetabulum into two anterior and posterior collecting tubules, thus agreeing with the condition that Jhonson (1920, p 353) considers to be the probable arrangement in the primitive *Echinostome cercaria*.

Closely allied to the Echinostomes there are certain other forms characterized among other features by the absence of collar spines in the cercaria, which Sewell has grouped together under 'Aglis' and 'Reflexa' groups. In the latter group the excretory system appears to resemble that of the second group of Faust and Sewell's 'Echinata' group, while the 'Aglis' group falls into line with 'Echinotoides' and 'Coronata' groups, in which the descending collecting tubule bifurcates at the level of the acetabulum.

The present species does not agree with any other known groups. In the general plan of the excretory system it resembles that of the 'Echinotoides' as exemplified in *Cercariae Indicae* XLVIII, Sewell, in so far as the following features are concerned: (a) The caudal canal is continued back to the posterior region of the tail. (b) The bladder is undivided into an anterior and posterior region. (c) The main collecting tubules enter the bladder close to the middle line on the anterior aspect. (d) The refractile granules contained in the main excretory tubule are few in number, and are restricted to that portion of the duct lying anterior to the acetabulum.

There are however differences in that the main collecting tubule does not appear to divide into ascending and descending tubules, the various flame-cells open either directly by short tubules or these tubules unite with other tubules, and open by a common secondary tubule into the descending limb of the main excretory tubule, also no side branches opening on the surface of lateral aspect of the tail could be detected such as are commonly present in the cercaria of the above groups.

In its general plan the excretory system of the body would appear to follow closely the arrangement described by Faust in *C. circumtricata* (*Parasitology*, Vol. XIV Nos. 3 and 4 1922 Plate XXI) (see Text-fig. 2c), though in this latter species the excretory tubule does not form a triangular flexure at the side of the pharynx.

It seems probable that in the course of evolution, the general character of the excretory system has undergone a progressive development. It commences with the type described and figured by Faust (1918, p. 100 Plate II, fig. 14) in *Cercaria achanthostoma* in which the main excretory tubule runs forwards receiving branches from flame-cells throughout the length of the body, and terminating at the side of the oral sucker in a triangular flexure (see Text-fig. 2d).

The same plan is followed in *C. chisolnata* and *C. trivialis*, but with this difference that the main collecting tubules are not dilated and full of granules, and the flame-cells and the tubules that originally opened into the duct have become suppressed. To compensate for this the main collecting tubule is in the next stage represented by *C. mehrui*, continued backwards from the triangular flexure receiving flame cells as it goes to the side of the excretory bladder.

In a subsequent stage this tubule forms two bends so that there run now three tubules up and down the body, and again the flame-cells open into the terminal part of the tubule, but in this case into the part that is running from the side of the oral sucker to the excretory bladder, i.e., the first descending loop, and finally in *Cercaria echinosomum revolutum* the main collecting tubule bifurcates at the level of the acetabulum into ascending and descending tubules into which the flame cells from the anterior and posterior halves of the body open.

**Locomotion**—Locomotion is performed in two different ways, when crawling the larva uses its suckers. The anterior end of the body is extended, and attachment is made by the oral sucker, the whole body is then drawn forwards to such an extent that the ventral sucker touches the oral sucker, the oral sucker is then released and a further attachment is again made. When swimming the cercaria uses its tail for locomotion. With the aid of lashing movements of the tail the body is set in motion. It moves round and round, and can thus travel short distances. When the animal is reversed so that the ventral surface of the body is uppermost it rights itself in a curious way. The anterior portion of the body is twisted in such a way that the oral sucker assumes its normal position and attachment is effected with it. The whole body is then twisted like a spiral bringing the ventral sucker downwards and attachment is at once made. The body thus assumes its natural position.

**Redia**—The cercaria develops into a redia. The redia is orange coloured, and in some cases the colour is deeper at the anterior end of the body than towards the posterior end. The body is dotted all over externally with small rounded

globules which are probably excretory in nature. They are found in masses or arranged in lines. There is very little difference in the contracted and in the expanded condition of the redia, because alterations in length are due only to the eversion of the pharynx, and a slight change in the anterior part of the body.

The mouth is situated at the anterior end and leads back into a well-developed pharynx. The anterior end is protrusible. A distinct collar is present at the anterior end. The pharynx leads into a long tubular stomach which is full of dark granules, this extends a little less than half of the length of the body. At about one-half of the total length of the body is a pair of locomotor organs.

TABLE III

*Measurements of redia of C. mehrari*

Length of body		Breadth of body posterior to l.c. organs		Breadth of body anterior to l.c. organs	Distance between l.c. organs and the posterior end
Expanded	Contracted	Expanded	Contracted		
1.03 mm	0.975 mm	0.167 mm	0.221 mm	0.197 mm	0.551 mm

*Note*—In every column of the above table only mean measurement is indicated.

Each redia contains a large number of cercariae in various stages of development. Some of them are fully developed, and are seen to be constantly moving inside the body-cavity. A birth-pore is present at the anterior region. Cercariae were actually seen to be coming out of the body with their anterior end first.

A number of rounded cells, the germ-cells, are present at the posterior region of the body of the redia.

I was unable to trace the excretory system of the redia.

*Systematic position*—There are certain characters of *Cercaria mehrari* which show resemblance to the 'Aglis' group, Sewell, 1922, while in others this cercaria differs markedly from this group, and exhibits similarities with the members of the 'Echinostome' group.

The features in which it resembles *Cercariae Indicae* XLI (Sewell, *Ind. Jour. Med. Res.*, 1922) are as follows:—

1. The surface of the body beneath the cuticle is crowded with cystogenous cells, which contain rod-like granules.

- 2 There are no collar spines
- 3 The ventral sucker is situated about one-quarter the length from the posterior end
- 4 Development occurs in rediae There are well-developed locomotor organs in the rediae
- 5 The pharynx of the redia is well developed, and is followed by a long stomach

The features in which this cercaria differs from the *Cercaria Indica* XLI are as follows —

- 1 Whereas in the Agilis group, to which the *Cercaria Indica* belongs, there is no trace of any oesophagus in *Cercaria mehuai* there is a short oesophagus leading back from the pharynx and terminating in a dilatation
- 2 The excretory bladder is not divided into an anterior and posterior chamber by a transverse constriction
- 3 The main collecting tubules arise close together near the middle line and not at the antero lateral angles of the excretory bladder
- 4 The gonad is represented by a mass of cells between the ventral sucker and the excretory vesicle which is probably the rudiment of the ovary and possibly the mass of cells anterior to the ventral sucker and behind the dilated end of the oesophagus is the rudiment of the vagina *Cercaria mehuai* resembles the members of the 'Echinostome' group in this particular respect
- 5 The posterior end of the tail tapers abruptly to a sharp point, as in *Cercaria Indica* XLVIII (Sewell), which belongs to the Echinostome group of Sewell, but there is no tail fin

I therefore conclude that this cercaria stands midway between the Echinostome and the Agilis group of Sewell

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ABBREVIATIONS USED IN PLATES LXXIV, LXXV AND LXXVI

*C.*, cercaria, CC, cystogenous cells, EG, excretory granule, EV, excretory vesicle, FC, flame-cell, G, gonad, GC, germ cell, LO, locomotor organ, MED, main excretory duct, OS, oral sucker, Ph and P Ph, prepharynx, R.ET, reflected excretory tubule, S, stomach, T, tail, V, vagina, VS, ventral sucker

PLATE LXXIV

O S

F C

R.E.T.

E.G.

M.E.D.

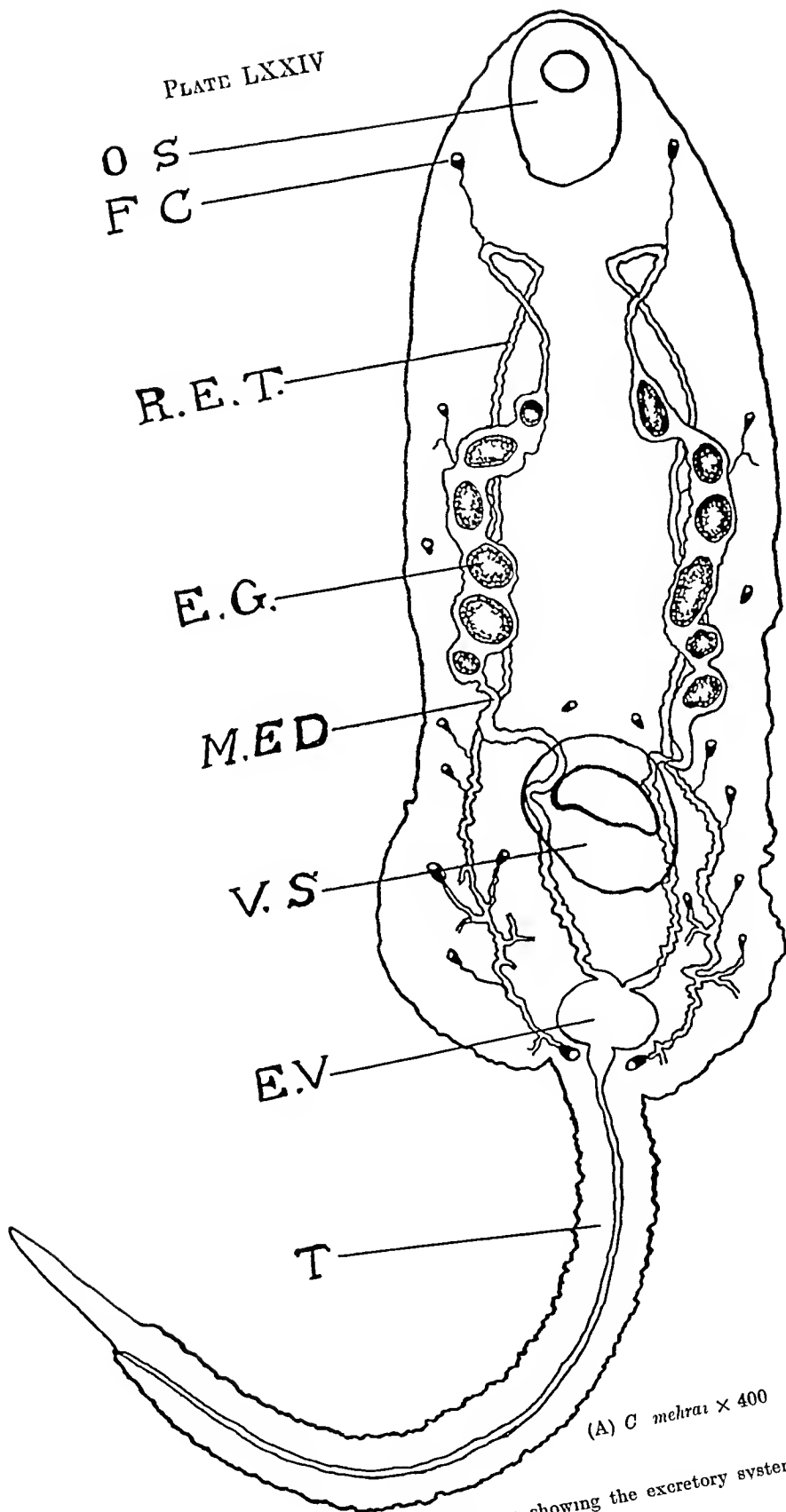
V.S.

E.V.

T

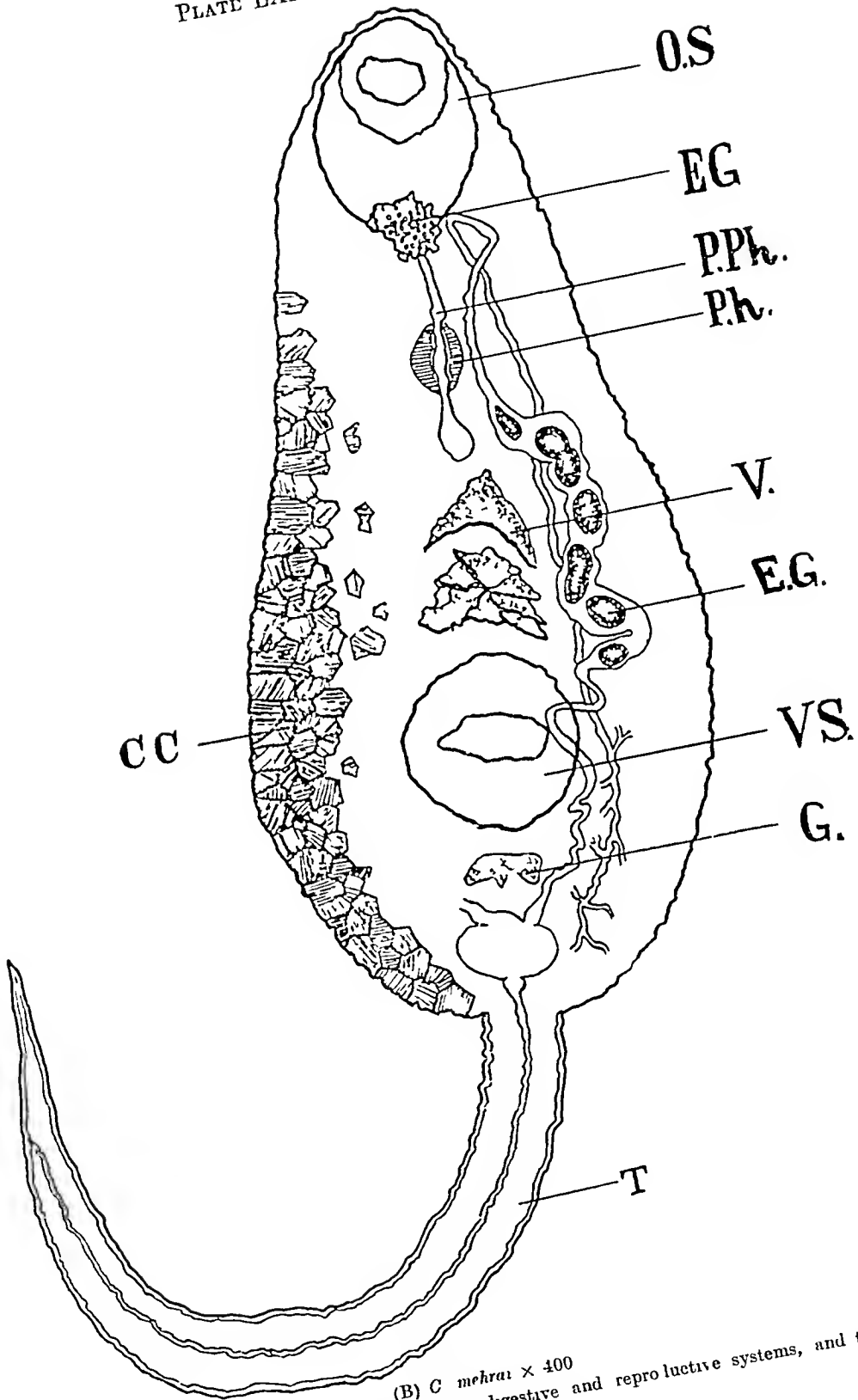
(A) *C. mehrai* × 400

*Cercaria mehrai* Ventral view showing the excretory system









(B) *C. mehrai*  $\times 400$   
*Cercaria mehrai* Ventral view, showing digestive and reproductive systems, and the arrangement of the cystogenous cells on one side only



# THE ACTION OF THE ACTIVATED SLUDGE PROCESS ON SEWAGE BACTERIOPHAGE

BY

LIFUT-COLONIL A D STEWART, M R , F R C S E , D P H , D T M & H , I M S ,  
*Professor of Hygiene,*

AND

S C GHOSAL, M R , D P H ,  
*Assistant Professor Public Health Laboratory Practice in Bacteriology, Calcutta  
School of Tropical Medicine and Hygiene*

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In a previous paper(1) we have shown that the activated sludge process has a definite germicidal action on different species of faecal organisms present in sewage. The process by which this purification is effected is very little understood. Different theories have been advanced by different workers, but are conflicting. Fowler(2) has expressed the view that purification by the activated process can be referred entirely to bacterial activity. Melling(3) and others say that it is mainly physical. Bushwell and Long(4) conclude that biological action is necessary, while Parson and Wilson(5) are of opinion that enzymic action is one of the important factors.

The remarkable rapidity with which this reduction is brought about and the fact that sewage contains bacteriophage capable of lysing faecal organisms, suggested an investigation as to whether the lytic action of sewage phage plays any part in this germicidal effect.

The experiments were designed to investigate the following points —

- (1) Whether bacteriophage originally present in the sewage increases in activity in the course of the activated sludge process and thereby cause such rapid diminution of bacteria, if not
- (2) What becomes of bacteriophage present

The problem was studied under the following varying conditions —

- (1) In an activated sludge plant at Hooghly Jute Mill, on the outskirts of Calcutta. This is an experimental plant with a system of air lift aeration.

(2) In a 'Simplex' activated sludge plant in a mill area near Calcutta

(3) In the laboratory, using aspiration apparatus for aeration

The experimental work dealt with a comparative study of the lytic action of the filtrates of raw sewage and the filtrates of its effluent after treatment, on the coli group of organisms, and on *B. shiga* and *flexner*

The group of *B. coli* organisms used in our experiments were isolated from mixed human stool, sewage, and polluted water, their identity being established by sugar tests

It may be mentioned that in our preliminary observations we found that filtrate of raw sewage we worked with was able to lyse about 95 per cent of all species of coli group of organisms. This agrees with the findings of Houston(6) and Caldwell(7). But our experience in this respect was more varied as we tested all possible faecal lactose fermenters isolated from different sources mentioned above, whereas Houston worked mainly with *B. coli communis* and Caldwell with pathogenic organisms isolated from infection of the urinary tract. We also found that sewage phage from Calcutta sewage and also from other sources mentioned above always lysed the strains of *B. shiga* and *flexner* used in our work

#### EXPERIMENTAL PROCEDURE

(1) Filtration of raw sewage and its effluent for isolation of bacteriophage

#### TECHNIQUE OF FILTRATION

Ten c.c. each of raw sewage or its effluent after treatment were put into 50 c.c. of nutrient broth (pH 7.8), incubated for 18 hours at 37°C and then filtered through filter paper coated with infusorial earth, and finally filtered through a L3 candle in Martins' filtering apparatus. The filtrates were kept in an incubator overnight for testing sterility and then stored in a cool room till used.

(2) The method of comparison of lytic action of filtrate of raw sewage with that of its effluent in each experiment was as follows —

Twenty-four hours broth cultures of organisms to be tested were made. Ten coli groups of organisms were generally used in each experiment. Two sets of broth tubes were then arranged. One loopful of each culture was inoculated into 7 tubes in each set. One was set aside as a control and the other six tubes in one set were inoculated with the filtrate of raw sewage to give dilutions of 1 in 10, 100, 1,000, 10,000, 100,000, 1,000,000. To the other set was added the filtrate of the effluent in the same manner. The broth tubes were then placed in the incubator at 37°C and examined for lytic action after 6 hours. One loopful from each tube in each set was then spread over agar slants to study 'plaque' formation.

To indicate the relative degree of lytic action possessed by filtrate of raw sewage as well as its effluent, we have used the following expressions —

— Indicates no lytic action of the filtrate on the given organism. A normal culture of bacilli develops in the broth or on agar. This shows that bacteriophage is absent.

+ Indicates weak lytic action The growth in the broth of bacteria to which the filtrate has been added is almost normal and transfer on agar slants shows a few plaques

+ - Shows moderate lytic action There is moderate growth in broth, transfer of this culture on agar shows larger number of plaques

+++ There is slight growth in the broth and transfer on agar shows mottled growth or a few colonies

++++ Indicates strong lytic action There is complete lysis No growth appears on transfer to agar

(1) Results of experiments with Hooghly Jute Mill plant

About six hours aeration was done here Out of a series of experiments conducted, the result of one is given below in Table I

TABLE I

Name of organisms	Dilution 1 in					
	10	100	1,000	10,000	100,000	1,000,000
1 <i>B. coli</i> communis (from water)	R S + + + +	+ + + +	+ + + +	+ + + +	—	—
	efflu + + + +	+ + + +	+ + +	—	—	—
2 <i>B. coli</i> communis (from sewage)	P S + + + +	+ + + +	+ + + +	+ + + +	++	—
	efflu + + + +	+ + + +	+ + + +	—	—	—
3 <i>B. coli</i> communis (from mixed human stool)	R S + + + +	+ + + +	+ + + +	—	—	—
	efflu + + + +	+ + + +	—	—	—	—
4 <i>B. schafferi</i> (from water)	R S + + + +	+ + + +	+ + + +	+ + + +	+ + + +	—
	efflu + + + +	+ + + +	+ + + +	+ + + +	—	—
5 <i>B. neopolitani</i> (from mixed human stool)	R S + + + +	+ + + +	+ + + +	+ + + +	+ + + +	++
	efflu + + + +	+ + + +	+ + + +	+ + + +	—	—
6 <i>B. neopolitani</i> (from water)	R S + + + +	+ + + +	+ + + +	+ + + +	—	—
	efflu —	—	—	—	—	—

R S = Raw sewage  
efflu = effluent after treatment

TABLE I—*contd*

Name of organisms	Dilution 1 in					
	10	100	1,000	10,000	100,000	1,000,000
7 <i>B. coscoroba</i> (from sewage)	R S + + + +	+ + + +	+ + + +	+ + + +	+ + + +	—
	efflu + + + +	+ + + +	+ + + +	++	—	—
8 <i>B. acid. lacti</i> (from mixed human stool)	R S + + + +	+ + + +	+ + + +	+ + + +	+ + + +	—
	efflu + + + +	+ + + +	+ + + +	+++	—	—
9 <i>B. vesiculosus</i> (from sewage)	R S + + + +	+ + + +	+ + + +	+ + + +	—	—
	efflu + + + +	+ + + +	+ + + +	—	—	—
10 <i>B. vesiculosus</i> (from mixed human stool)	R S + + + +	+ + + +	+ + + +	+ + + +	+ + + +	—
	efflu + + + +	+ + + +	+ + + +	+ + + +	+ + + +	—
11 <i>B. shiga</i> (labora- tory strain)	R S + + + +	+ + + +	+ + + +	+ + + +	+ + + +	—
	efflu + + + +	+ + + +	+ + + +	+ + + +	—	—
12 <i>B. flexner</i> (labo- ratory strain)	R S + + + +	+ + + +	+ + + +	+ + + +	+ + + +	—
	efflu + + + +	+ + + +	+ + + +	+ + + +	—	—

R S = Raw sewage  
efflu = effluent after treatment

It will be seen from this table that there is a definite decrease in lytic action of sewage bacteriophage in the course of this process. There has been 90 per cent reduction of activity against *B. shiga* and *flexner*. But it will be noticed that activity against all the coli group of organisms has not been reduced in the same proportion. Roughly, here also there is about 90 per cent reduction in most of the cases. Out of 10 coli organisms tested, activity has decreased in 9 cases. In one it has remained unaltered. In another there is no lysis even in 1 in 10 dilution.

Viewing the result of series of experiments conducted with this plant, it may be stated that compared with the raw sewage, the effluent showed reduction of about 90 per cent in activity of sewage phage against *B. shiga* and *B. flexner*. In the coli group of organisms tested there was reduction in most. The percentage of reduction was variable, but was 90 per cent in the majority of cases. Activity against some of the organisms remained unaltered while in a few destruction of phage was practically complete.

## (2) Results with Titagar plant

The results obtained here corroborated in the main our previous findings. The results are practically a repetition of those given above.

## (3) Laboratory experiment

The experiments conducted here were mainly directed to find out the rate of decrease in activity of sewage phage.

Raw sewage was mixed with 20 per cent activated sludge and aerated for periods varying from 1 to 24 hours. After aeration, the sewage was allowed to settle and the supernatant effluent was taken out hourly up to the end of 6th hour and also after 24 hours.

A control was done where the sewage only was aerated without any addition of activated sludge. No reduction took place in the activity of sewage phage in the control.

The rest of the procedure was as described above.

In these experiments we found no consistent decrease in lytic action of sewage phage before 6 hours' aeration.

The result of 6 hours' and 24 hours' aeration is given below in Table II.

TABLE II

Name of organisms	Dilution 1 in						
		10	100	1,000	10,000	100,000	1,000,000
1 <i>B coli communis</i>	A	++++	++++	++++	++++	++++	—
	B	++++	++++	++++	++++	—	—
	C	++	+	—	—	—	—
2 <i>B coli communis</i>	A	++++	++++	++++	++++	++++	++
	B	++++	++++	++++	++++	—	—
	C	—	—	—	—	—	—
3 <i>B coli communis</i>	A	++++	++++	++++	++++	+	—
	B	++++	++++	++++	—	—	—
	C	++	—	—	—	—	—

A = Raw sewage B = Effluent after 6 hours' aeration

C = Effluent after 24 hours' aeration

The organisms used in this experiment are same as in Table I



TABLE II—*contd*

Name of organisms	Dilution 1 in						
		10	100	1,000	10,000	100,000	1,000,000
4 <i>B schafferi</i>	A	++++	++++	++++	++++	++	—
	B	++++	++++	++++	—	—	—
	C	+	—	—	—	—	—
5 <i>B neopolitanus</i>	A	++++	++++	++++	—	—	—
	B	++++	++++	—	—	—	—
	C	—	—	—	—	—	—
6 <i>B neopolitanus</i>	A	++++	++++	++++	++++	—	—
	B	++++	++++	++	—	—	—
	C	—	—	—	—	—	—
7 <i>B coscoroba</i>	A	++++	++++	++++	++++	++++	—
	B	++++	++++	++++	++++	—	—
	C	+	—	—	—	—	—
8 <i>B acidilacti</i>	A	++++	++++	++++	++	—	—
	B	—	—	—	—	—	—
	C	—	—	—	—	—	—
9 <i>B vesiculosus</i>	A	++++	++++	++++	++++	++++	++
	B	++++	++++	++++	++++	—	—
	C	+	—	—	—	—	—

A = Raw sewage    B = Effluent after 6 hours' aeration  
C = Effluent after 24 hours' aeration  
The organisms used in this experiment are same as in Table I

TABLE II—*concl'd*

Name of organisms	Dilution 1 in						
		10	100	1,000	10,000	100,000	1,000,000
10 <i>B. vesiculosis</i>	A	++++	++++	++++	++++	++++	—
	B	++++	++++	++++	++++	—	—
	C	—	—	—	—	—	—
11 <i>B. typha</i>	A	++++	++++	++++	++++	++++	++
	B	++++	++++	++++	++++	—	—
	C	++	—	—	—	—	—
12 <i>B. flexner</i>	A	++++	++++	++++	++++	++++	—
	B	++++	++++	++++	++++	—	—
	C	+	—	—	—	—	—

A = Raw sewage B = 1 fluent after 6 hours' aeration

C = 1 fluent after 24 hours' aeration

The organisms used in this experiment are same as in Table I

It will be seen that the results of 6 hours' aeration agree with those in Table I.

After 24 hours' aeration the sewage phage has practically disappeared. Where the filtrate of the effluent failed to lyse in the lowest dilution we determined whether the phage was completely destroyed or only attenuated.

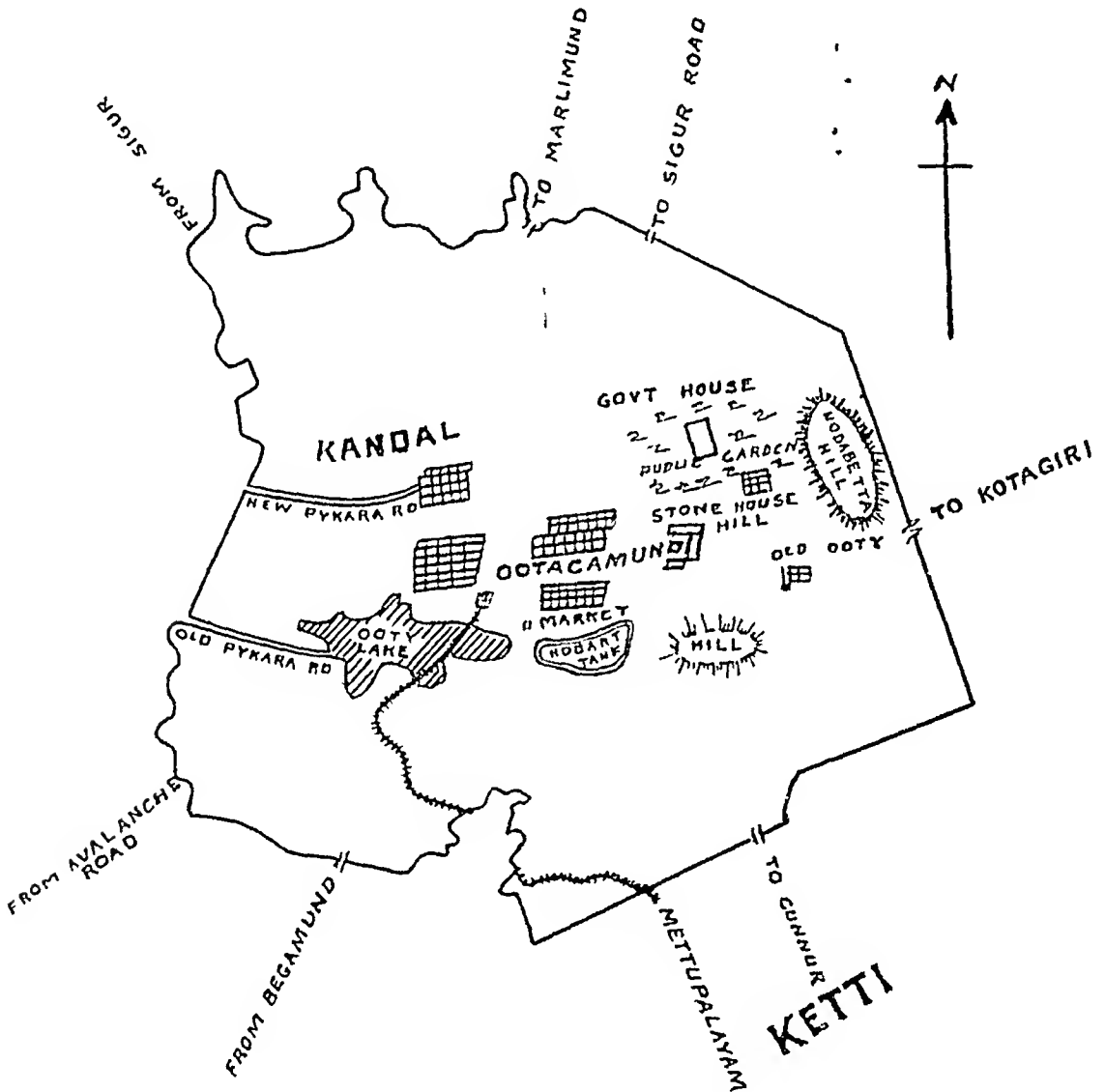
For this purpose, the strain was submitted to a few passages with the given bacteria. In some cases the bacteriophage was recovered, in others we failed to recover phage after five passages showing that it had been completely destroyed.

### DISCUSSION

In a previous paper we have shown that in the activated sludge process, coli as well as pathogenic organisms in the sewage diminish at a definite rate from the first hour. Phage would appear to be more resistant than bacteria. There was little or no reduction with less than 6 hours' aeration. Even then lytic action against some of the coli group of organisms remained unaltered. The explanation

The warmest month is April, and the coolest January. The average maximum temperature in April is about  $7^{\circ}\text{F}$  higher than the average maximum in January. The average minimum temperature of April is about  $9^{\circ}\text{F}$  higher than the average minimum in January. The average maximum temperature is  $65.6^{\circ}\text{F}$

MAP OF OOTACAMUND SHOWING ONLY THE CROWDED RESIDENTIAL AREAS



and the average minimum is  $49.5^{\circ}\text{F}$ . Annual average rainfall is 49 inches, nearly half of it being during the South-West monsoon from June to September. Generally speaking, Ootacamund possesses a temperate and an equable climate and ranks very high amongst the hill stations of India.

The meteorological observations made during the period of the survey are tabulated below —

TABLE I

Place	Period of survey	Mean dry bulb temperature 8 A M in Fahr	Mean wet bulb temperature 8 A M in Fahr	Humidity per cent	Mean saturation deficiency in inches	Average annual rainfall in inches
Ootacamund	May-June 1929	62.0°	57.5°	77.0	0.13	49

*Housing conditions sanitation etc* —The middle class houses are all crowded together in Kandhal, old Ootacamund and Vannarapettai, while the houses of the upper classes are distributed irregularly, each with its own extensive grounds. The houses are well constructed and do not afford much shelter for rats. The floors of houses are usually covered with coir mattings or planks of wood. These, however, harbour enormous numbers of *Pulex irritans*, which are indeed a pest in this town.

The granaries and godowns are situated mostly by the side of the market and in the bazaar, some of these are rat proof.

The general sanitary condition of the town is excellent.

*Plague* —Plague first occurred in an indigenous form in June 1903, and it reappeared almost every year till 1910. From 1910 to 1915 the town was free from it, but in 1915 to 1916 there was a mild attack. There was no further outbreak till 1922. In 1922 to 1923 there was again a small outbreak, and since then the town has been free from plague. It may be mentioned here that in 1922, there occurred in the town an outbreak of pneumonic plague, with 6 attacks and 5 deaths.

Ketti had plague first in 1913 to 1914 and again in 1926 to 1927.

Ever since 1903, plague has been appearing every year in epidemic proportions in one part or another in this district.

The survey was done in May to June, the summer season of the district. Ketti was also surveyed. Every representative area was selected, but since the results were practically uniform in all areas the figures have been tabulated only under two heads, *Ootacamund* and *Ketti*.

*Rodents* —Two hundred and twelve rodents were examined during the survey, and out of these 125 were *R. rattus*, 61 *Gunomys lok*, 14 house mice, 9 musk rats and 3 bandicoots.

The wonder traps, which are being used in this survey, were not found to be quite serviceable here. This has been also the experience of the Municipal plague staff. The back-spring wooden trap (locally called the break-back trap) gave better results. These traps were also particularly useful for trapping field rats.

These, baited with potatoes, were either buried in rat holes or places along rat runs. Attempts were also made to get as many live samples of field rats as possible, but only 8 of them could be so caught mostly in bandicoot traps. The following Table shows the varieties of rodents examined, with their sex distribution —

TABLE II  
*Rodents obtained with their sex distribution, etc*

Variety of rat	Total number caught	Females per cent	Number caught alive
<i>R. rattus</i>	125	54.4	88
<i>Gunomys lok</i> (field rat)	61	55.7	8
Mouse	14	42.8	3
Musk rats	9	55.5	
Bandicoots	3	66.6	3
TOTAL	212		102

It is thus seen that the females were in slight excess in all cases except in the case of mice. *R. rattus* were mostly of the white-bellied variety. Specimens of the field rats—*Gunomys lok*—were sent to the Bombay Natural History Association and we are indebted to them for their identification. These swarm into houses only during the off-season for potato cultivation. They predominated among the rodents trapped in wooden traps, forming 61 out of 124 specimens. Since the wooden traps were equally useful for all other rodents, it may be said that *Gunomys lok* is the predominating rodent in Ootacamund. Mice and musk rats also exist in large numbers in houses.

Table III gives the rat density, etc., for *R. rattus* in Ootacamund and Ketti.

TABLE III  
*Rat density, etc*

Places	Number of traps laid	Number of <i>R. rattus</i> caught	Number of <i>R. rattus</i> per 100 traps or rat density	Female <i>R. rattus</i> per cent	Pregnant to total females per cent	Average number of foetus es	Replenishment rate for 100 rats per day
Ootacamund	1,583	77	5	54.4	14.8	5	4.4
Ketti	110	11	10	60.0			

Rat density is thus found to be very low in Ootacamund. As was reported by the Plague Commission the rat density in 1911 was also very low, viz., 6 (*Journal of Hygiene*—Plague supplements Volumes XIII to XV, 1914—17). There has also been no change in rat density during all these years in spite of the fact that systematic rat catching is being done in this Municipality for over 20 years. It is to be noted too that the replenishment rate for rats here is the same as is found in other parts in this Presidency.

All the rodents caught were autopsied, and their spleen smears examined for *B. pestis* with negative results. It was observed that about 60 per cent of the field rats, and about 20 per cent of the house rats showed some kind of coccidial infection of the liver. Further, no specimen showed any chronic abscesses, scars on perisplenitis indicating lesions of chronic plague infection.

*Fleas*—Four hundred and twenty-seven rat fleas were collected, from Ootacamund and Ketti. Out of these 379 were from *R. rattus*, 22 from *Gunomys* *lol*, 11 from mice and 15 from bindicoots. The following were the species, in the order of their prevalence on *R. rattus*—

<i>Xenopsylla cheopis</i>	19	per cent
<i>Ceratophyllus</i> (Species ?)	22.6	„
<i>Xenopsylla brasiliensis</i>	11.5	„
<i>Xenopsylla astia</i>	9.7	„
<i>Leptopsylla musculi</i>	3.7	„
<i>Pulex irritans</i> —one specimen from a mouse		

It was observed that *X. cheopis* was the chief flea on *R. rattus*, *Ceratophyllus* on *Gunomys lol* and *L. musculi* the chief flea on mice. Bindicoots mainly harboured only *X. astia* though one of them yielded one *X. cheopis* also.

The following Table shows the general and specific flea indices for *Gunomys lol*—

TABLE IV

*Flea indices for Gunomys lol*

Variety of rat	Number of specimens	Total fleas collected	General flea index	Specific <i>Ceratophyllus</i> index	Specific <i>X. cheopis</i> index	Specific <i>X. brasiliensis</i> index
<i>Gunomys lol</i>	8	22	2.75	2.5	0.25	0.25

Table V shows the general and specific flea indices, etc., for *R. rattus* in Ootacamund and Ketti—

TABLE V  
Flea indices, etc., for *R. rattus*

Place	Total fleas collected	General flea index	Specific <i>X cheopis</i> index	Specific <i>Cerato phyllus</i> index	Specific <i>X brasiliensis</i> index	Specific <i>X astia</i> index	Specific <i>L. musculi</i> index	SEX DISTRIBUTION OF FLEAS				
								<i>X cheopis</i> females per cent	<i>Cerato phyllus</i> females per cent	<i>X brasiliensis</i> females per cent	<i>X astia</i> females per cent	<i>L. musculi</i> females per cent
Ootacamund	276	3.6	1.75	1.0	0.2	0.5	0.11	54.8	54.6	49.0	62.1	57.1
Ketti ..	103	9.3	4.60	0.7	3.5		0.45					

It is to be noted that the general flea index for Ketti is about 3 times as high as that for Ootacamund and here no *X. astia* was found in 103 fleas examined. Specific indices for *X. cheopis* and *X. brasiliensis* are also very high in Ketti. This is significant, for this village had severe epidemics of plague, one as recently as 1927.

### DISCUSSION

The interesting feature of this survey was the variety of the flea fauna. It was mentioned that among the different species of fleas, *X. cheopis* and *Ceratophyllus* predominated. Both of these are recognized efficient vectors of plague. It is also known that in the case of *C. fasciatus* the optimum temperature required for effective transmission of the infection is about 15° lower than that required in the case of *X. cheopis*.

These factors might have an important bearing on the seasonal periodicity of plague in the locality. Perhaps in summer the conditions are favourable for *cheopis* fleas and in winter for *Ceratophyllus*, and so the epidemic and epizootic plague may be more or less continuous. Lieut.-Col. A. J. H. Russell, in his hitherto unpublished note 'The Geographical Distribution of Plague in the Madras Presidency,' mentions that the plague epidemics in the Nilgiris District show no marked variation between one season and another, while the death rate is highest in October to December and least in March to April.

The probable rôle of the field rat in the epidemiology of plague in Ootacamund is to be noted. These rats form the chief rat menace in the town, being present in alarming numbers around houses and in the open. During the season for potato cultivation, they keep to the fields but in the off-season they swarm into houses. It has already been stated that they harbour in fairly large numbers *Ceratophyllus* (specific index 2.5) which flea might be an efficient vector of plague. They must be kept under control. Perhaps a more practicable measure would be to restrict potato cultivation around dwellings.

The abundance of *Pulex irritans* on the floors of houses is to be noted. As Dr. J. W. H. Chun has shown, during the Manchurian plague epidemic these can successfully transmit the infection. One has therefore to emphasize the need of scrupulous house sanitation to diminish the pest of these fleas.

### SUMMARY

(1) The survey was done in May and June—the summer season of the station.

(2) In all 212 rodents were examined during the survey. *R. rattus* and *Gunomys tal* were the predominating species of rats found.

(3) The density for *R. rattus* was very low, viz., only 5 for every 100 traps laid, but for Ketti—a village close by—it was found to be 10.



(4) The rats trapped were examined for signs of plague with negative results

(5) Four hundred and twenty-seven fleas from rats were examined. The general flea index was 3.6

The following were the species found arranged in the order of their prevalence on *R. rattus*, with their specific flea indices —

<i>X. cheopsis</i>	1.75
<i>Ceratophyllus</i>	1.0
<i>X. brasiliensis</i>	0.2
<i>X. astia</i>	0.5
<i>Leptopsylla musculi</i>	0.11
<i>Pulex irritans</i>	.

The general and specific flea indices for Ketti were about 3 times as high as those for Ootacamund

## REPORT NO. V.

### A RAT-FLEA SURVEY OF THE TWO MARITIME TOWNS—MADRAS\* AND NEGAPATAM †

#### I MADRAS

MADRAS, the head-quarters of this Presidency, is the third largest city in India and has a population of 526,911. It is nine miles long along the sea coast, and has an average breadth of three miles. It is low-lying and flat. The Cooum river winds across it, dividing it into a northern and southern range. The Buckingham canal runs from north to south, and separates the coastal division from the rest of the town. Although Madras has no natural harbour, yet from the value of its trade, it ranks high among the sea ports of India. George Town is the commercial centre of the city. Parts of the city like George Town, Chinadripet and Triplicane are much over-crowded. The grain godowns are mostly situated in George Town and in Wall Tax Road in Park Town. There are two big cotton mills in the city one at Perambur, and the other at Choolai situated within a mile of each other.

Saidapet, a municipal town in the Chingleput district is about 5 miles distant from Madras, but from the point of view of a plague survey, it may be taken as a suburb of Madras.

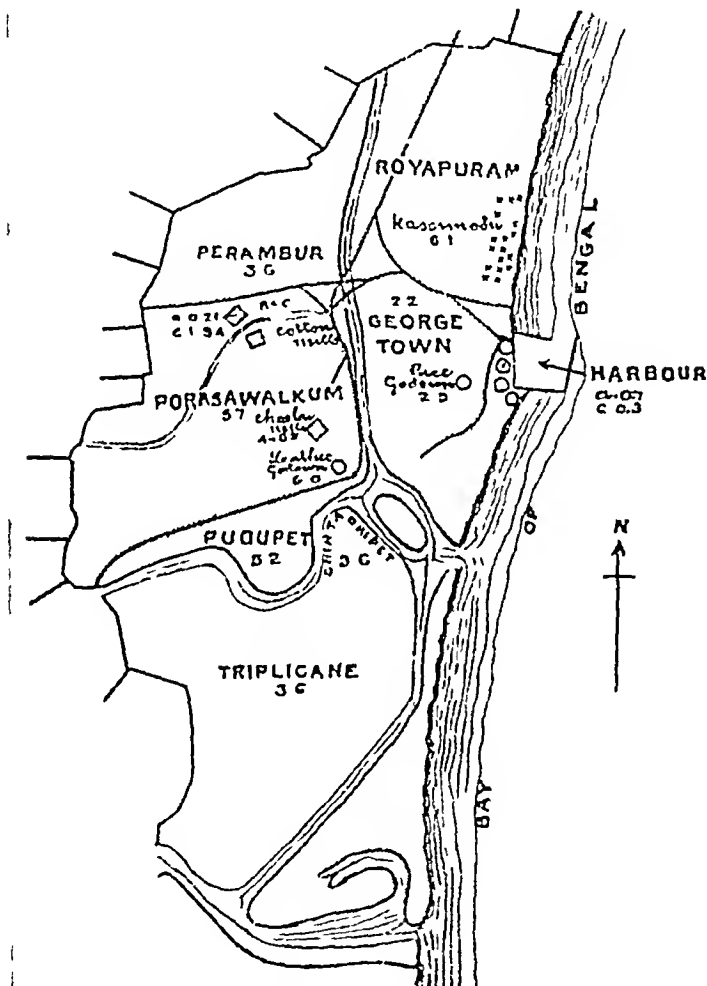
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\* This place was surveyed by Drs. P. V. George and D. S. Mankikar.

† This place was surveyed by Dr. D. S. Mankikar.

*Climate* —The climate of the place is generally hot. The data regarding the monthly temperature, saturation deficiency and rainfall is averaged for over a period of 15 years (1910—25) is discussed later. The meteorological observations

MAP OF MADRAS CITY SHOWING FIPA INDICES IN DIFFERENT AREAS



*Note* —Figures represent the maximum *X. astia* index, where *X. cheopis* are also found. Separate flea indices for both are given

□ Cotton mills    a = *X. astia*  
○ Godowns       c = *X. cheopis*

made during the period of survey, with the average annual rainfall, are tabulated in Table I

TABLE I  
*Meteorological observations*

Place	Period of survey	Mean dry bulb temp erature 8 A M in Fahr	Mean wet bulb temp erature 8 A M in Fahr	Humidity per cent	Mean saturation deficiency in inches	Average annual rainfall in inches
Saidapet	March 1929	78.8°	73.4°	77	0.24	51
Madras	July-August 1929	85.0°	74.6°	60	0.45	51

*Risk of Plague* —Madras stands the risk of importation of plague, both by sea and land. Although Madras has only an artificial harbour, there are facilities for ships to come along the quay side and discharge their cargo. The harbour is in frequent communication with Rangoon, which, as is known, is infected with plague.

By land Madras is linked up with the rest of the Indian Peninsula by two railway systems. A northern line connects Madras with Calcutta, a southern line with Tuticorin, a north-west line with Bombay and a south-west line with the west coast of the Presidency and Mysore. Thus it is brought into close communication with several important foci of infection.

*Housing conditions* —The Royal Commission on plague investigations in India, in their seventh report, describes the conditions in Madras City thus: —‘It is difficult in a few words to convey any idea of the house construction in the native quarters of the city such as Madras when one sees all types of dwelling from the simple mud hut with mud floor and tiled or thatched roof to the solidly built two-storied dwelling of the well-to-do inhabitants. Let it suffice to say that from the point of view of rats and plague, the best houses, built as they are on high stone plinths, would be difficult to improve on, whereas the poorer houses afford unlimited shelter for rats. There certainly appears to be nothing in the construction of large portions of the city, that can explain its immunity from plague, and as we shall presently see, rats are present in abundance everywhere.’ Conditions have changed little since then, and these remarks are apt even to-day.

*Grain godowns* —There are no rat-proof godowns in the city either within the harbour or outside it. However, the godowns in the harbour are all provided with concrete or Cuddapah slab flooring and corrugated zinc sheet roofing and further they are well lighted, ventilated and kept clean. The godowns within the city offer a different picture altogether. They are all crowded together in George Town, very near the harbour and are constructed and maintained under conditions which afford unlimited shelter for rats and also provide facilities for active flea breeding.

*Plague* —There has been only one indigenous outbreak of plague in the city in 1905. This was confined to a few villages on the northern outskirts of the city in Kassimodu. The source of the infection was either from the Fiji-Mauritius

immigration depot which was then situated close to this area, or from the harbour, through the coolies who formed a colony in one of these villages. The infection smouldered on for about four months. Some of these villages were burnt down, and efficient measures were also taken in and around the other villages close by which soon brought the infection to a close. Madras has been free from indigenous plague since.

However imported cases of plague are being notified almost every year since 1900 with the exception of 1902 1903 1904 1915 and 1919, giving an average death-rate of 0.01 per mille for 20 years from 1906 to 1925.

### THE SURVEY

The Madras survey was done in the summer July-August 1929, the survey of Saidapet was done two months earlier i.e. in March-April 1929.

The city is divided into 30 municipal divisions. Instead of surveying all the divisions an attempt was made to survey only the representative areas in the city. The figures obtained have been tabulated as two sets, one set consolidated to the various types of areas and the other to the different divisions for future comparison.

*Rodents*—In Madras 1,025 rodents were examined out of which 23 were bandicoots and the remaining 1,002 were *R. rattus*. In Saidapet 378 rodents were examined out of which 11 bandicoots and the remaining 367 were *R. rattus*. It may be mentioned that in Saidapet one mongoose was trapped together with 9 rats, and in Madras a toddy cat was also caught in the trap.

It was interesting to find in the harbour certain varieties of *R. rattus* which were not met with anywhere in the city, e.g. in some ventral and dorsal fur was jet black in colour and in others the dorsal fur was of a rusty red and the ventral fur of a lighter red colour.

A comparison of the rodent distribution in Madras City in 1910 during the survey of the Royal Commission on plague, and that found during the present survey is attempted below—

TABLE II  
*Rodent distribution*

Period of survey	Total collection of rodents	<i>R. rattus</i> per cent	Bandicoot per cent	Mouse per cent	Musk rat per cent	Number of <i>rattus</i> for 100 traps or <i>R. rattus</i> density	REMARKS
1910 Jan—Dec	17,663	49.4	1	46.1	3.5	25	The figures in the last column give the <i>R. rattus</i> density for corresponding periods in the year.
1929 July—Aug	1,025	97.8	2.2			29	

It will be seen from the Table that no mice were trapped during the present survey while in the 1910 survey they formed nearly 46 per cent of the total rodents obtained. Of course different traps were employed during the two surveys but this fact alone cannot explain the difference, since with our traps mice and musk rats were readily trapped in all our surveys elsewhere. However, as one may expect, there is no corresponding great change in the *R. rattus* density, as seen from the last column of above Table.

Table III gives details regarding rat density, etc. in different representative areas. Similar details for the various municipal divisions are given in Appendix I.

TABLE III  
*Rat density, etc., for different types of areas in Madras City*

Areas	Number of traps laid	Number of rats caught	Number of rats per 100 traps or rat density	Female <i>R. rattus</i> per cent	Pregnant <i>R. rattus</i> to total females per cent	Replenishment rate for 100 rats per day *
Harbour	1,241	206	17	53.3	26.3	
Rice godowns in George Town, near harbour	96	94	97	58.5	47.2	
Bazaar areas	572	319	56	58.6	14.4	
Residential areas	535	139	26	59.7	14.5	
Kassimodu fishermen's huts	96	22	23	54.5		
Alwatherry	48	54	113	46.3	2.8	
Leather godowns in New Town	25	5	20	60.0		5.3
Cotton mills at Perambur	200	47	24	51.1	33.3	
Area around the cotton mills at Perambur	200	46	23	54.3	40.0	
Cotton mills at Choolai	160	35	22	51.8	14.3	
Area around the cotton mills at Choolai	53	11	21	63.6		

\* The rate of replenishment is got by multiplying the percentage of pregnant females to total rats by the average number of foetuses and dividing by the number of days pregnancy is grossly visible in rats which is taken on an average as 16 days. This method of calculation is according to the formula published in *U. S. A., P. H. Reports*, Vol XLIV, 1929, No. 9.

Table IV gives details for rat density, etc., in the different areas in Madras harbour —

TABLE IV

*Rat density, etc., in Madras harbour*

Areas in the harbour	Number of traps laid	Number of rats caught	Number of rats per 100 traps or rat density	Female <i>R. rattus</i> per cent	Pregnant females to total females per cent
Rice godowns	597	91	15	53.3	26.3
Ground nut godowns	501	87	17		
Barges	113	28	20		
<b>TOTAL</b>	<b>1,211</b>	<b>206</b>	<b>17</b>	<b>53.3</b>	<b>26.3</b>

It is to be noted that the godowns in George Town, which is the commercial centre of the city gave the highest figure (97) for rat density (except Alwarcherry), and the godowns in harbour gave the lowest figure (17). This remarkable difference is worthy of special emphasis and may be easily explained by the differences in the construction and upkeep of these godowns.

Just as elsewhere the female rats predominated. All the rodents examined were autopsied, and spleen smears examined. The pregnancy rate, the average number of foetuses, and from these data the replenishment rate of rats, were all calculated.

*Fleas*—Two thousand seven hundred and sixty-six rat-fleas were examined in Madras, of which 2,608 were *Xenopsylla astia*, 154 *X. cheopis*, 2 *X. brasiliensis*, 1 *Pulex irritans*, and 1 *Ctenocephalus felis*. In Saidapet 1,179 fleas were examined and all were *X. astia*. Thus it is evident that *X. astia* is the indigenous flea of Madras and suburbs, while the others are only recent emigrants into the place. The distribution of *X. cheopis* in the city is at present patchy, since they were found only in the harbour, in the rice godowns of George Town, and in cotton mills at Perambur. In the harbour they formed 30 per cent and in the cotton mills at Perambur 90 per cent of the total fleas, while in George Town only 3 specimens were obtained from the godowns stocking Rangoon rice. Two specimens of *X. brasiliensis* were obtained from the ground-nut godowns in the harbour. The source of importation of these fleas will be discussed later.

1236 *A Rat-Flea Survey of Madras and Negapatam Towns*

Table V gives the general and specific flea indices, etc., for the different types of areas in the city. The results for the various municipal divisions are given in Appendix II.

TABLE V

*Flea indices, etc. for the different types of areas in Madras City*

Areas	Number of fleas collected	General flea index	Number of <i>X. astia</i>	<i>X. astia</i> index	Number of <i>X. cheopis</i>	<i>X. cheopis</i> index	SEX DISTRIBUTION		REMARKS
							Female <i>X. astia</i> per cent	Female <i>X. cheopis</i> per cent	
Harbour	213	1.03	150	0.73	60	0.29	55.0	56.0	2 <i>X. brasiliensis</i> and 1 <i>Pulex irritans</i>
Rice godowns in George Town near harbour	268	2.88	265	2.85	3		61.3	66.2	
Bazaar areas	796	2.49	796	2.49			57.2		
Residential areas	516	3.71	516	3.71			55.8		
Kassimodu fisher men's huts	136	6.10	136	6.10			54.4		1 <i>Ctenocephalus felis</i>
Alwarcherry	135	2.50	135	2.50			53.3		
Leather godowns in New Town	30	6.00	29	5.80			53.3		
Cotton mills at Perambur	101	2.15	10	0.21	91	1.93	50.0	56.0	
Area around cotton mills at Perambur	178	3.32	178	3.32			47.2		
Cotton mills at Choolai	170	4.82	170	4.82			46.2		
Area around cotton mills at Choolai	46	4.30	46	4.30			41.3		

Table VI gives details of flea indices, etc., for the different areas in the harbour —

TABLE VI

*Flea indices etc for the different areas in Madras harbour*

Areas	Number of fleas collected	General flea index	Number of <i>X. astia</i>	<i>X. astia</i> index	Number of <i>X. cheopis</i>	<i>X. cheopis</i> index	SEX DISTRIBUTION		REMARKS
							Male <i>X. astia</i> per cent	Female <i>X. cheopis</i> per cent	
Rice godowns	98	1.08	48	0.53	50	0.55	55	56	
Ground nut godowns	108	1.24	98	1.12	8	0.09			
Barges	7	0.25	1		2				1 <i>Pulex irritans</i>
<b>TOTAL</b>	<b>213</b>	<b>1.01</b>	<b>150</b>	<b>0.73</b>	<b>60</b>	<b>0.29</b>	<b>55</b>	<b>56</b>	

It may be noted from Tables V and VI that the lowest flea index (1.03) was noted in the harbour. This is explained from the fact that all the godowns here have good flooring, they are all well-lighted and cleanly kept and provide only limited facilities for flea breeding.

The highest flea indices were met with in Kassimodu, and the leather godowns in New Town, viz., 6.1 and 6 respectively. Here again the explanation is not far to seek. Kassimodu is a village which consists of several hundreds of huts, occupied by fishermen and harbour coolies. The roofs as well as the sides of these huts are made of coconut palm leaves. The mud floors are invariably riddled with rat holes. Sanitation here is very poor and thus the available conditions are ideal for active flea breeding.

*Sex distribution of fleas*—Among both *X. astia* and *X. cheopis* the females formed a slightly larger percentage in all areas, except in and around the cotton mills, where among *X. astia* the males predominated.

*Grains and grain movements*—The city depends for all the grains consumed within it on imports from other places. Thus rice is imported mainly from Rangoon and Saigon by sea and Nellore and Guntur by rail. Wheat is brought mostly from Karachi, gram, dhol, and other pulses come from Rangoon, Karachi, Bombay

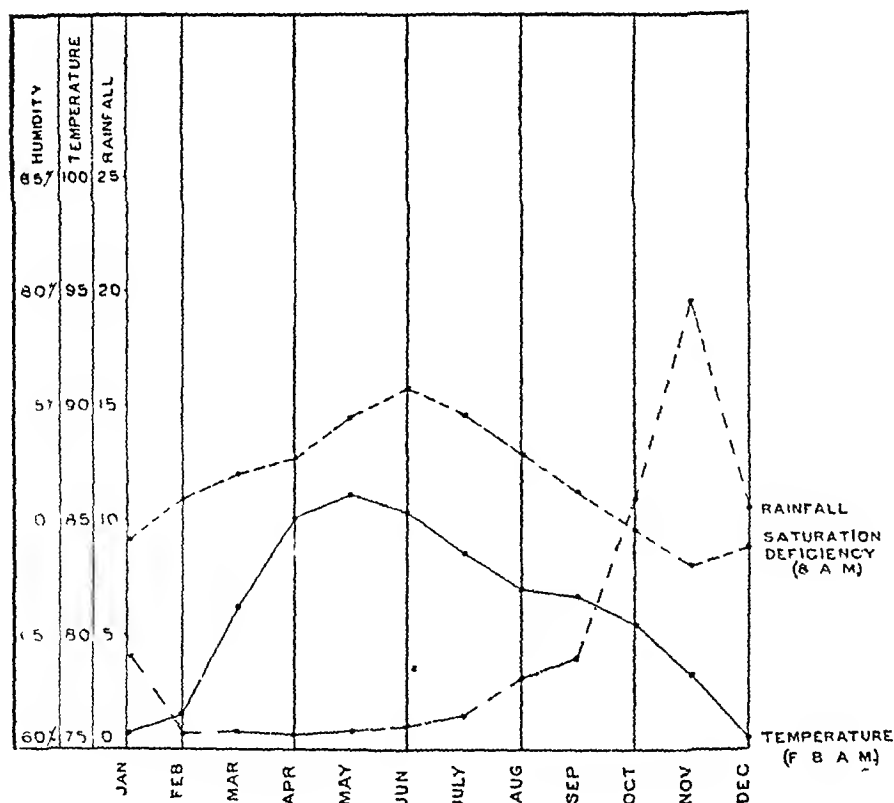


supply is so gathered ginned and delivered only after June (summer crop) Since the period of picking and ginning of the winter crop corresponds roughly to the period which is most favourable for flea breeding, this crop is likely to be infected with fleas more than the summer crop

Whether these factors alone will serve to explain the difference in the two mills we do not know, but observations recorded here regarding cotton clearly show that

### NEGAPATAM

*Graph showing the seasonal incidence of rainfall temperature and humidity averaged over the period 1910—1925*



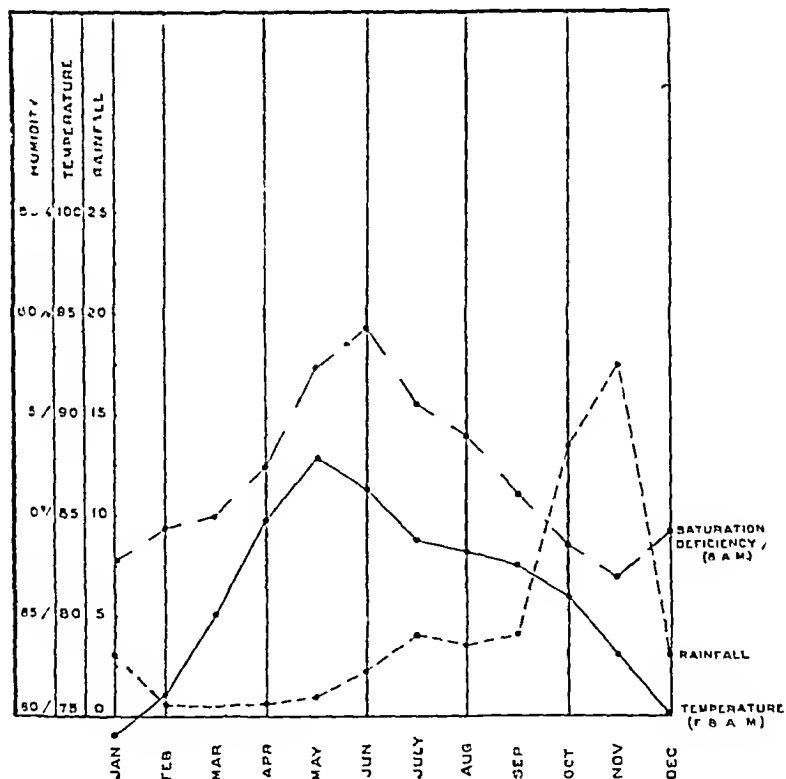
it is a favourable medium for the dispersal of fleas, and further emphasize the importance of the seasonal factor in such importation

The results of the flea survey at Negapatam are described in detail in Part II of this report As is shown then the only species of rat flea obtained there was

*A. astia* This total freedom of Negapatnam from *X. cheopis* cannot be explained easily. Both Madras and Negapatnam are important seaports on the East coast of this Presidency. Both are in direct and frequent communication with Rangoon, and large quantities of Rangoon rice are being imported into these places and

### MADRAS CITY

*Graph showing the seasonal incidence of rainfall temperature and humidity averaged over the period 1910—1925*



also into Colombo. While *X. cheopis* has established itself in Madras (30 per cent in the harbour area) and in Colombo, apparently it has failed to obtain a foothold in Negapatnam. The climatic conditions of the two ports are analysed below. Graphs I and II show the seasonal incidence of rainfall temperature

(8 A M) and saturation deficiency averaged for a period of 15 years—1910 to 1925. The figures for this purpose were obtained from the Madras Observatory. The climate of Negapatam would thus appear to be not very different from that of Madras.

There is one point, however, which has to be noted. In Madras harbour facilities have been provided of late for ships to be at anchorage along the quay side to discharge their cargo. About 5-12ths of the cargo that is brought to the harbour is thus unloaded directly on to the docks. The ships at Negapatam, however, lie at anchorage about 3 miles off the shore and goods are brought ashore in barges and lighters. This factor at any rate would limit the introduction of fleas to some extent. Whether it would serve to give complete protection against such importation it is difficult to say.

#### SUMMARY

1 During the survey 1,025 rodents were examined. Out of these 23 were bandicoots and the rest *R. rattus*. In Saidapet 378 rodents were examined and out of these 11 were bandicoots and the rest *R. rattus*. The general rat density was also high, viz., 29.

The godowns in George Town, the bazaar areas in all divisions of the city and the slums in Alwarcherry harbour a very large rat population.

2 In Madras, 2,766 rat fleas were examined, of which 2,608 were *Xenopsylla astia*, 154 *X. cheopis*, 2 *X. brasiliensis*, 1 *Pulex irritans*, and 1 *Ctenocephalus felis*. In Saidapet 1,179 fleas were examined. They were all *X. astia*.

3 There is evidence to show that *X. cheopis* is being gradually introduced into the city. At present it is found mainly in the harbour area and in the cotton mills in Perambur. In the case of the harbour such introduction probably takes place through rice imported from Rangoon and in the case of cotton mills in Perambur through cotton brought to the mills from other *X. cheopis* infected places in and outside the Presidency. The seasonal factor in such importation is discussed.

4 The general and the specific flea indices for all areas are however low, that for *X. cheopis* being less than 2 and for *X. astia* less than 4, except for leather godowns and Kassimodu which is nearly 6.

5 The susceptibility to plague of the Madras rats is indeed well recognized. From the point of view of the introduction of infection, a set of highly disturbing factors are found to exist here, viz., the highly susceptible rat, the gradual invasion by the most efficient plague vector, *X. cheopis*, and a frequent communication with Rangoon, one of the principal foci of infection among the maritime cities of the Orient. Judging from the trend of similar events in Colombo in 1914, it must be pointed out that early preventive measures are indicated if the present freedom of the city from plague is to be maintained.

## APPENDIX I

*Rat density etc. for the different places in Madras and Saidapet*

Places	Number of traps laid	Number of rats caught	Number of rats per 100 traps or rat density	Female rats per cent	Pregnant rats to total females per cent	Replenishment rate for 100 rats per day
Harbour	1,311	206	17	53.3	26.3	53
Rice godowns in George Town	96	94	97	58.5	47.2	
Residential areas in George Town	114	31	21	64.5	15.0	
Muthalpet bazaar	96	82	85	51.2	9.5	
Broadway "	48	16	33	56.2		
Alwaracherry	48	51	113	46.3	2.8	
Royapuram Kassimodu	96	22	23	54.5	8.3	
Chintadripet bazaar	96	80	83	68.8	20.7	
" residential area	48	16	33	81.3	15.4	
Parl Town Wall Tax Road godowns	18	14	92	74.4	40.6	
" bazaar	18	20	42	70.0	14.3	
" residential area	48	6	13	66.0		
Kotuvai Chavadi bazaar	88	13	16	38.0	25.0	
" residential area	44	10	23	75.0		
Purasawalkam bazaar	84	42	50	64.3	18.5	
" residential area	42	11	26	63.6	28.6	
Perambur bazaar	133	44	33	45.5	20.0	46
" cotton mills	200	47	24	51.1	33.3	
" around "	240	46	19	55.8	37.5	
Choolai bazaar	97	35	36	54.3	10.5	
" cotton mills	160	35	22	51.4	11.1	
New Town leather godowns	25	5	20	60.0		
Puthupet	47	14	30	71.4	10.0	
Triphicane bazaar	86	34	39	67.6	21.7	
" residential area	53	18	33	38.9		
Saidapet	1,451	378	26	69.8	21.5	
TOTAL	4,907	1,403				

## APPENDIX II

*Flea indices, etc., for R. rattus for the different places in Madras and Saidapet*

Places	Number of fleas collected	General flea index	Number of <i>X astia</i>	<i>X astia</i> index	Number of <i>X cheopis</i>	<i>X cheopis</i> index	SEX DISTRIBUTION		REMARKS
							Female <i>X astia</i> per cent	Female <i>X cheopis</i> per cent	
Harbour	213	1 03	150	0 73	60	0 29	55 0	56 0	2 <i>X brasiliensis</i> 1 <i>Pulex irritans</i>
Rice godowns in George Town	268	2 88	265	2 85	3		61 3	66 2	
George Town residential area	90	2 2	90	2 2			61 1		
Muthalpet bazaar	271	3 3	271	3 3			52 7		
Broadway "	73	4 5	73	4 5			63 0		
Alwarcherry	135	2 5	135	2 5			53 3		
Royapuram Kassi-modu	136	6 1	136	6 1			54 4		
Chintadripet bazaar	70	0 9	70	0 9			61 4		
" residential area	58	3 6	58	3 6			60 3		
Park Town Wall	155	3 5	155	3 5			56 1		
Tax Road godowns									
" bazaar							69 1		
" residential area							55 0		
Kotuvai Chavadi bazaar	20	1 7	20	1 7			55 0		
" residential area	7	0 9	7	0 9			57 1		
Purasa wakka m bazaar	109	2 6	109	2 6			52 3		
" residential area	63	5 7	63	5 7			46 0		
Perambur bazaar	158	3 6	158	3 6			49 3		
" cotton mills	101	2 15	10	0 21	91	1 93	50 0	56 0	
" around "	178	3 3	178	3 3			47 2		
Choolai bazaar	130	3 7	130	3 7			40 0		
" cotton mills	170	4 8	170	4 8	..		46 2		
New Town leather godowns	30	6 0	29	5 8			53 3		1 <i>Ctenocephalus felis</i>
Puthupet	112	5 2	112	5 2			58 9		
Triplacine bazaar	80	2 4	80	2 4			58 8		
" residential area	64	3 6	64	3 6			57 8		
Saidapet	1,179	3 1	1,179	3 1			50 7		
TOTAL	3,945		3,787		154				

In calculating the indices, the fleas obtained from bandicoots have been strictly omitted

## II NEGAPATAM

NEGAPATAM, a municipal town in the Tanjore district, is about 270 miles south of Madras and is a seaport of considerable importance on the Coromandel coast, being the port of call of ships from Rangoon, Straits and Karachi. It is

connected by a branch line to the main railway line of the South Indian Railway running from Madras to the south and forms the distributing centre of grain and merchandise to other parts of the Madras Presidency

The climate is hot and dry. The average annual rainfall is 54 inches. The meteorological observations made during the period of the survey are tabulated below —

*Meteorological observations*

Place	Period of survey	Mean dry bulb temperature 8 A.M. in 1 hr	Mean wet bulb temperature 8 A.M. in 1 hr	Humidity per cent	Mean saturation deficiency in inches	Average annual rainfall in inches
Negapatam	June-July 1929	81.00	76.00	68.0	0.42	54.00

The population is 51,016. Within the Municipality, the chief divisions are Negapatam town proper, the port, Vellipalayam, Kadambady, and Nagore. The port is about a mile from the town proper—Nagore forms the northern portion of the town and is about three and a half miles away from Negapatam. The railway line runs through all these places and Nagore forms the railway terminus. There is as yet no proper drainage system and sanitation on the whole is poor.

*Housing conditions*—Better class houses built on modern style are all situated in Kadambady. The houses in the town, Vellipalayam and Nagore are mostly single storied with roofs made up of coconut rafters covered with country tiles. The roofs therefore afford excellent facilities for the nesting of rats. All areas are congested except Kadambady.

The godowns are all situated in the port area. These are not rat proof. The floors are only thinly cemented and very often found to be riddled with rat holes. Grain is stored in dark and ill-ventilated rooms and the conditions here are not unfavourable for rat and flea breeding.

*Plague*—There was only one indigenous outbreak of plague in 1913-14 with 154 attacks and 141 deaths. This was mostly limited to the harbour area. It is to be noted that Colombo also became affected in 1913. According to Hirst, 'the ultimate source of the infection of both Negapatam and Colombo was undoubtedly Rangoon which became first infected in 1905' (*Researches on Parasitology and Plague*, Vol. I, part 5, 1927, page 344). *X cheopis* has now become established in Colombo while in Negapatam as will be seen from the present survey as well as that of Major Cragg in 1922 that not a single *X cheopis* was seen amongst the fleas examined.

The survey was done during June-July 1929, which forms perhaps the hottest part of the year in the place.

*Rodents*—Altogether 466 rodents were trapped Out of these 456 were *Rattus rattus*, 9 bandicoots and one 'musk rat' The *R rattus* were all of the brown-bellied variety with the exception of one white-bellied specimen The following Table shows the number of traps laid, the density and the sex distribution of rats —

*Rat density, etc*

Place	Number of traps laid	Number of rats caught	Number of rats per 10 traps or rat density	Per cent female rats	Per cent pregnant females to total females	Replenishment rate for 100 rats per day
Negapatam bazaar	178	88	49	61.3	27.8	3.2
Nagore „	144	79	54	62.0	25.0	
Negapatam residential area	274	43	15	72.0	6.5	
Nagore „ „	153	43	28	58.1	12.0	
Port „ „	116	38	32	65.7	28.0	
Vellipalayam „ „	270	49	18	71.4	20.0	
Kadambady „ „	54	4	7	25.0		
Port godowns	242	108	44	66.6	29.2	
Negapatam railway goods shed	54	12	22	75.0		
Nagore railway goods shed	27	2	7	50.0		

All the rats trapped were autopsied Out of 301 female rats dissected, 69 were pregnant and the average number of their foetuses was 3.4 The replenishment rate (for 100 rats per day) of rats in the locality was also calculated from the above data and found to be 3.2 It will be noticed from the table that the godowns and bazaar areas harbour a large rat population with a highest rat density of 44 and 54 respectively

*Fleas*

*X astia* was the only rat flea found Out of 1,680 fleas examined, 792 or 47 per cent were females The flea index was comparatively low in all the areas probably owing to the fact that the survey was done during the summer season The flea index for the godowns in the port area was found to be the highest (4.4) It may be mentioned that the only 'musk-rat' trapped showed 6 *X astia* on it

*Fleas and flea indices*

Place	Total fleas collected	General flea index	<i>X. astia</i>		<i>X. astia</i> index	REMARKS
			Total	Per cent females		
Negapatam bazaar	296	3.1	296	35.8	3.4	In calculating flea indices the fleas collected from bandi- coots have been excluded in all cases
Nagore "	269	3.4	269	45.2	3.4	
Negapatam residential area	85	1.5	85	61.2	1.8	
Nagore "	101	2.3	101	54.5	2.3	
Port "	168	3.0	168	51.2	3.0	
Vellipalavam "	231	3.8	231	43.3	3.8	
Kadambadi "	20	0.3	20	55.0	0.3	
Port godowns	478	4.4	478	50.8	4.4	
Negapatam railway goods shed	30	2.5	30	56.7	2.5	
Nagore railway goods shed	2	1.0	2		1.0	

*Grain movement*

The imports and exports of Negapatam are both by sea and land. The ships anchor off some miles away in the sea and the goods are brought to the harbour in barges and lighters. Grains are imported into Negapatam mainly for distribution to the surrounding districts by rail and partly for local consumption. Thus rice (broken and raw) is imported from Rangoon, Cocanada and Puri—gram, dholl and other pulses come from Rangoon, Bombay, and Karachi—wheat and wheat flour are largely imported from Karachi. Penang sends gunnies, areca nuts, tapioca and sago. Ground-nuts are brought in large quantities from surrounding districts to Negapatam for export.

Although rice forms the chief import into Negapatam it is also exported in large quantities to Penang, Singapore and Port Swettenham.

## SUMMARY

Four hundred and sixty-six rodents were trapped, of these 456 were *R. rattus*, 9 bandicoots and 1 'musk rat'. The conditions in the granaries and bazaars were found to be very favourable for rat and flea breeding.

(2) One thousand six hundred and eighty rat-fleas were collected in Negapatam. All these belonged to the species *X. astia*.



(3) Both Colombo and Negapatam are in constant communication with Rangoon and the presence of *X cheops* in the former and its complete absence in the latter port is to be noted

## REPORT NO VI.

### A RAT-FLEA SURVEY OF NELLORE,\* TIRUPATI,\* TIRUMALAI† AND TANJORE †

#### I NELLORE

NELLORE is a municipal town with a population of 37,000. It consists of a number of suburbs close to one another, the most important from the point of view of this survey, being the Stonehousepet to the north of the town.

All the rice mills are situated in this area excepting two which are in other parts of the town. The main street in this suburb is the Stonehousepet bazaar. This street contains all the godowns and shops which stock grains imported from various places particularly the Nizam's State. All other shops selling rice and grain in retail are situated in a street at right angles to the bazaar only a few yards away.

Nawabpet is another suburb in continuation of the bazaar of Stonehousepet and contains among other quarters the houses and shops of those who stock and press gingelly seeds for oil.

In the heart of the town proper there is another bazaar street. The shops here get their supply of grains from the Stonehousepet godowns.

The climate of the place is to be described as hot and dry. The mean maximum from 8th April to 3rd May was 97.2°F in the shade and the minimum was 78.7°F. The mean wet and dry bulb readings taken at 8 A.M. were 78.1°F and 84.3°F the saturation deficiency being 0.35 inches.

#### *Export and import*

The main export is rice. It forms about 80—85 per cent of the total exports of the town. About 2,000,000 bags of rice are exported annually to all the southern and western districts of the Madras Presidency. The bags are generally despatched

\*        - places were surveyed by Dr. N. Natarajan  
†        - place was surveyed by Dr. P. V. George

straight from the rice mills and it is very rare for them to be stocked in the Stonehousepet godowns before being sent out

Castor nut is another item of small export forming about 10 per cent of the export, the export being to Cuddapah and Chittoor districts

Import is mainly from the Nizam's Dominions Various food-stuffs such as green gram, Bengal gram, black gram horse gram, castor seeds, coriander seeds, cashu nut wheat gingelly seeds, mustard, and large quantities of chillies are imported in considerable quantities—on the average of about 24,000 bags annually Gummy bags are got from Vizianagar and coco nuts are imported from Calicut

*Rats*—The species prevailing here is *Rattus rattus* *Rattus norvegicus* and mice were not noted White-bellied rats formed about 12 per cent of the rats trapped One thousand and forty seven rodents were trapped of which four were bandicoots Fifty rats were taken for flea breeding experiments The rest were examined for fleas Out of 997 rats examined, 709 or 67.7 per cent were females The figures for rat density, etc., are detailed in Table I —

TABLE I

*Rat density, etc*

Places	Number of traps laid	Number of rats per 100 traps or rat density	TOTAL NUMBER OF RATS CAUGHT			Number of pregnant females	Number of fetuses found
			Males	Females	TOTAL		
1 Stonehousepet bazaar	133	156	73	135	208	42	177
2 Stonehousepet rice mills	54	133	21	51	72	8	41
3 Stonehousepet domestic quarters	51	128	25	44	69	13	64
4 Nawabpet	42	162	18	50	68	7	34
5 Ranganayakulupet	54	87	15	32	47	9	30
6 Kapadipalayam	54	37	7	13	20	4	20
7 Nellore, west	54	57	6	25	31	7	28

1250 *A Rat-Flea Survey of Nellore, Tirupati, Tirumalar and Tanjore.*

TABLE I—concl'd

Places	Number of traps laid	Number of rats per 100 traps or rat density	TOTAL NUMBER OF RATS CAUGHT			Number of pregnant females	Number of fetuses found
			Males	Females	TOTAL		
8 Nellore, east	54	85	15	31	46	11	48
9 Moolapet	54	19	3	7	10	1	4
10 Fatekhanpet Police lines	42	62	13	13	26	2	11
11 Fatekhanpet domestic quarters	28	118	4	29	33	6	32
12 Parallel roads between Nellore east and bazaar	54	72	10	29	39	7	28
13 Nellore town bazaar	107	188	72	129	201	31	153
14 Grand road Trunk road	39	118	10	36	46	7	35
15 Domestic quarters	53	89	12	35	47	9	41
16 Durgammet	42	79	13	20	33	4	18
17 Railway goods shed	49	2	1		1		

There was a large density of rat population as evidenced by the number of rats trapped per 100 traps laid both in the domestic and bazaar quarters, the latter as usual having a larger rat population. The density was 87 rats per 100 traps laid for the domestic quarters and 170 rats per 100 traps laid for the bazaar areas. If the domestic quarters in the vicinity of the Stonehousepet bazaar were alone considered, the density rises considerably viz., 139 per 100 traps laid while for domestic quarters away from the bazaar area it falls to 71 per 100 traps laid.

*Fleas*—Thirteen thousand four hundred and five fleas were examined. Table II below gives the results—

TABLE II  
*Fleas and flea indices*

Place	Total fleas examined	<i>X. astia</i>		<i>X. cheopis</i>		<i>X. astia</i> index	<i>X. cheopis</i> index
		Males	Females	Males	Females		
1 Stonehousepet bazaar	878	231	244	212	161	2.2	1.9
2 Do rice mills	210	117	122		1	3.3	
3 Do domestic quarters	275	132	179	1	3	3.9	
4 Nizampet	209	98	110	1		3.1	
5 Ranganayakulpet	116	48	68			2.5	
6 Kapadipalayam	106	53	53			5.3	
7 Nellore, west	107	51	53			3.5	
8 Nellore, east	161	61	98	1		3.5	
9 Moolpet	54	23	31			5.4	
10 Fatekhanpet Police lines	60	25	35			2.3	
11 Do domestic quarters	121	45	76			3.7	
12 Parallel roads between Nellore east and bazaar	123	49	74			3.2	
13 Nellore town bazaar	593	180	165	133	115	1.8	1.2
14 Grand Trunk road	89	46	36	2	5	1.8	
15 Domestic quarters	208	98	110			4.4	
16 Durgammet	59	28	31			1.8	
17 Railway goods shed	4	1	3			4.0	

The rats from the bazaar areas only harboured both *X. astia* and *X. cheopis* while the rats trapped from other areas harboured only *X. astia*. It is to be noted that the Stonehousepet bazaar which imports and stocks grains from the Nizam's Dominions has a larger *X. cheopis* index (1.9) than the other bazaar areas in the town which only obtain their supplies from the former (1.2).

The general flea index was 3.4, 2.7 was the *X. astia* index while the *X. cheopis* index for the whole town was 0.8 but this last figure is fallacious because the rats of areas other than the two bazaar streets harboured no *X. cheopis*. Therefore if these two areas alone were considered the *X. cheopis* index per rat would be 2.89. 5 per cent of the total fleas were *X. astia*. The percentage would have been

## III TANJORE

TANJORE City, the head-quarters of the district of the same name, has a population of 60,341. It consists of the fort and two suburbs Karunthattangudi and Manambachavady. It is an important railway junction on the main line of the South-Indian Railway and is at a distance of 218 miles from Madras and 226 miles from Tuticorin.

*Climate* —The meteorological observations made during the period of survey are tabulated below —

TABLE I

Place	Period of survey	Mean dry bulb temperature 8 A.M. in Fahr	Mean wet bulb temperature 8 A.M. in Fahr	Humidity per cent	Mean saturation deficiency in inches	Average annual rainfall in inches
Tanjore City	April 1929	85.0°	79.00°	77.0	0.26	36.00

*Housing condition, sanitation, etc* —The city is an old historic town with a fort and a temple. The houses in the fort and other congested areas have a solid foundation and are terraced. However they were found to harbour numbers of rats.

The godowns are mostly situated in the East gate bazaar and Ayankadai. These are dark, ill-ventilated rooms where rats find ideal shelter both in the flooring and in the ceiling.

The arrangement for the disposal of rubbish is not satisfactory, and the drainage and general sanitation is very poor. The city is in most parts so overcrowded that enforcement of adequate sanitation is beset with great difficulties.

*Plague* —The city has never had plague in an indigenous form. Imported cases were reported in 1905, 1910, 1914 and 1919. In 1919 a few rat falls were also reported in the Hospital compound. The district as a whole also has been free from plague except Negapatam which had an outbreak in 1913-14.

*Survey* —The survey was done in April, at about the height of summer in the locality. Every representative area in the city was selected and also a village Papanasam, about 15 miles from Tanjore.

*Rodents* —Seven hundred and fifty-six rodents were examined. Out of these 699 were *Rattus rattus*, 46 *Rattus norvegicus*\*, and 11 bandicoots. *Rattus rattus* were mostly of the brown-bellied variety. A few dark-bellied ones were also obtained but none of the white-bellied type. *Rattus norvegicus* were obtained mainly from houses and were often trapped together with *R. rattus*. The distribution of this species was not confined to any particular part of the town. Bandicoots were almost always got from Manambachavady, where they seem to

\* These are provisionally identified as such

abound in large numbers. The figures of rat density, sex distribution, etc., are detailed in the following Table —

TABLE II

*Rat density, etc*

Places	Number of traps laid	Number of rats caught	Number of rats for 100 traps rat density	Per cent female rats	Per cent pregnant females to total females	Average number of foetuses per pregnant rat	Replenishment rate for 100 rats per day
TANJORE							
Bazaar areas —							
East gate bazaar	196	271	138	55.5	20.1	5.3	3.8
Avanladai „	121	80	66				
Clock tower „	176	81	60				
Vandipettah „	89	49	55				
Residential areas —							
Fort	197	102	52	55.5			
Manambachivadi	119	113	42				
Karunthittangudi	82	26	32				
Papanasam	20	14	70				

The females were slightly in excess. All the rodents were autopsied, and spleen smears examined for *B. pestis* with negative results. In the females the number of foetuses whenever present was also noted, and from this the replenishment rate of the rats was calculated.

Rat density was high in all areas in the city, but particularly in the grain godowns in the East gate bazaar where it was the highest (138). This figure is more than twice that of any other figure got in the city, and so reveals the conditions under which these godowns are constructed and maintained.

*Fleas*—Two thousand four hundred and five fleas were examined, all these were *Xenopsylla astia*, with the exception of one *Ctenocephalus felis*. The female fleas formed only 48.5 per cent of the total. The flea indices for *R. rattus*, for different areas in the city, are shown in Table III. The flea index for bandicoots was 14.2, and that for *R. norvegicus* 4.4.

TABLE III.

*Fleas and flea indices for R. rattus*

Places	Total fleas examined	General flea index	Total <i>X. astia</i>	Per cent female <i>X. astia</i>	<i>X. astia</i> index	REMARKS
TANJORE						
<i>Bazaar areas</i> —						
East gate bazaar	707	2.60	707	48.5		In calculating flea indices, only the fleas collected from <i>R. rattus</i> have been made use of
Ayankada „	230	2.87				
Clock tower „	282	3.48				
Vandipettah „	369	7.53				
<i>Residential areas</i> —						
Fort	223	2.18				
Manambachavady	480	2.47				
Karunthattangudi	85	3.27				
Papanasam	29	2.00		62.07		

The specific *X. astia* index for *R. rattus* which is also the general flea index, ranged from 3.48 for the clock tower bazaar to 2.18 for houses in the fort area, but it was as high as 7.53 in Vandipettah.

It may be interesting here to point out, that on comparing the flea index and rat density figures in both the bazaar and residential areas, it is found that the flea index diminishes as the rat density increases. There is, however, no definite

quantitative relation. Anyhow it may be deduced that the combined effect of the prevailing conditions of climate, housing and sanitation are more in favour of rats than rat-fleas.

*Grain movement*—Tanjore is one of the most fertile districts in the Presidency watered by the river Cauvery, and large quantities of rice, cholam, ragi, etc., are grown. Tanjore town gets its supply of grains, partly from within the district, and partly from other places. Thus the chief imports are the following—

Paddy comes almost entirely from surrounding villages. Rice from Rangoon, Nellore and Guntur. Wheat from Karachi and Bombay. Cholam, ragi, cambu, dholl, etc., from surrounding villages and also from Panruti and Cuddalore. Oil-seeds from Bombay Presidency, Hubli, Gokak and Bijapur. The chief exports are ground-nuts, paddy, dholl and gram.

The local grains are brought to the town by road, in country carts. These are first taken to the Vandipettah, where the gunnies are emptied, and grains sifted and remeasured to local dealers. The grains from Rangoon, Karachi, and Bombay are brought here by rail from the seaports of Negapatam, Tuticorin and Madras.

Attempts were made to find out if there was any gross infestation of the grains with rat-fleas or their larvæ. Samples of different grains, beatings from the gunnies and sweepings from the godowns were collected. Each sample was examined, extraneous larvæ, etc., removed, suspicious flea larvæ isolated and incubated in a sterilized mixture of bran, gunny dust, etc., and observed in the laboratory under suitable conditions. Twenty-six different samples were observed. It may be said at the outset that no adult fleas were detected in any samples. But in three samples 1 adult flea, all *X. astia*, emerged after incubating for about 4 weeks. Two of these samples were from grains just brought to the town, one being husk from rat-destroyed paddy in a gunny, and the other gunny dust. The third was from a sample of sweepings from the floor of a godown.

As would be seen from Tables II and III, Vandipettah shows a rat density and flea index of 55 and 7.53 respectively. As was mentioned before, Vandipettah receives all the grain supply from outside, before such grains are redistributed to local dealers. The area of this Pettah is perhaps not more than 2 acres. The high density and an equally high flea index evidently points to the frequent importation of fleas with such grains.

It is, however, curious that in spite of some grain trade with such *X. cheopis* infested areas as Hubli, Gokak and Bijapur, no introduction of *X. cheopis* has so far been noted in this locality.

#### SUMMARY

Seven hundred and fifty-six rodents were examined of which 699 were *R. rattus*, 49 *R. norvegicus*, and 11 bandicoots.

The sanitary and housing conditions of this city favour a large rat population.





# PREPARATION OF A POTENT ANTI-PLAGUE SERUM IN INDIA

BY

B P B NAIDU M D (Edin), M H D PH, D T M (L'pool),

JAMIDAR SHAMSHER JUNG I M D

AND

K H KAMAKAKA M R C P (Edin), D P H (Lond), D T M (L'pool)

*Haffkine Institute Parel Bombay*

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## INTRODUCTION

ALTHOUGH successful results following the administration of serum have been recorded from time to time from countries outside India, yet, the results which have attended the use of various anti-plague sera in our Plague Hospitals, have been considered as unsatisfactory. In 1913 the Advisory Committee on Plague Investigations(1) reported that from their inquiry 'the administration of the available sera is not a practical means of bringing about any material diminution in the mortality from plague, it may well be that better results will be obtained, if the treatment could be commenced within a few hours of the onset of the disease, this, however, is in a great majority of cases impossible in ordinary hospital practice'.

In 1923, Choksy who has an unrivalled experience in the treatment of this disease, contributed a series of articles to the *Times of India*(2) urging the necessity for undertaking researches into anti-plague serum in India, as 'it is the only remedy that holds out any hope of reducing the excessively high case mortality which has so markedly characterized the epidemics at Bombay'. These articles attracted considerable public attention. Therefore, Col Mackie, the Director of the Haffkine Institute, obtained the sanction of the Indian

Research Fund Association to proceed with researches into anti-plague serum, on the conclusion of an inquiry into anti-plague vaccines which was then in progress. In 1927, experiments on the possibility of preparing a potent anti-plague serum in India were commenced, and this paper deals with (A) the history of anti-plague serum, (B) the results which have hitherto attended the use of various anti-plague sera in man, (C) our attempts at the preparation of anti-plague sera in animals which are susceptible to experimental infection with one or other of the organisms of the pasteurilla group, and (D) the comparative results which have followed their use in Laboratory animals experimentally infected with plague, along with Yersin's anti-plague serum manufactured at the Pasteur Institute, Paris.

#### (A) HISTORY OF ANTI-PLAGUE SERUM

Following the discovery of the plague bacillus, Yersin, Calmette and Borrel(3) demonstrated the possibility of immunizing rabbits against plague and showed that the serum possessed both preventive and curative properties when tested in rabbits, encouraged by these results, they immunized a horse against plague and found that its serum also possessed preventive and curative properties in mice. These successful results made them hope that serum therapy could be applied to the plague-stricken man. Therefore, Yersin at Nha-Trang, and Roux, Calmette and Borrel at the Pasteur Institute, Paris, began the immunization of horses and as soon as a sufficient quantity of serum became available, Yersin(4) proceeded to Canton and then to Amoy and treated in all 26 cases with serum, of these only 2 died with a case mortality of 7.6 per cent. The publication of these results appeared about the time when plague broke out in Bombay. Yersin's results with serum were so striking that the Health Department of the Municipality of Bombay instructed Haffkine to undertake at once the preparation of serum. In November 1896, he began the immunization of horses, cattle, goats and sheep, on a plan similar to that announced by Yersin, in February 1897, he(5) tested the serum at the Plague Hospital, Bombay, on six patients who were in a serious condition and consequently not likely to recover, with six similar cases for controls. Out of the six treated, three died and of the six controls five died with a percentage mortality of 83.3, the three among the treated who succumbed, lived longer than the untreated, and out of the four cases treated within 24 hours of the onset of the disease, only one died. From these results Haffkine concluded that with the further immunization of animals, it might be possible to obtain a serum which may have a favourable influence on late cases. In October 1897, when he had accumulated sufficient amount of serum, he(6) treated every alternate case admitted to the Poona Plague Hospital, out of the two hundred cases which passed through his hands, one half were treated with varying amounts of serum, the other half served as controls. From the clinical observations, he obtained no evidence of any

favourable effect attributable to the serum and the case mortality among the treated was some 11 per cent higher than among the controls. As his results with serum were entirely of a negative character, he abandoned its further preparation. The next attempt to prepare serum locally was made in November 1898. Lustig prepared anti-plague serum by injecting horses with plague nucleo-proteid which proved to be curative in experimental animals, this serum was sent to Bombay in 1897 for trial. By the time it had arrived, the epidemic had run its course and only a few sporadic cases were available for treatment. Choksy who was in charge of the Arthur Road Hospital, where it was first tried, reported that it was 'the only serum which gave anything like satisfactory results, as out of the 23 cases treated with it only five succumbed with a case mortality of 21.7 per cent'. On the strength of this report, the Corporation agreed to the local production of Lustig's serum and engaged the services of Lustig's assistants Drs. Galeotti and Polverini, for the purpose. This serum had an extensive trial for over two years and as the results following its use were not more favourable than those which attended the use of other sera, the Corporation discontinued its preparation in June 1902. This is the second and last attempt at the production of anti-plague serum in Bombay. Apart from these two locally prepared sera, other anti-plague sera sent out to Bombay have also been tried in our hospitals. The first of these is the anti-plague serum of Yersin, on the recommendation of Lord Lister, the Secretary of State desired that the Government of India should invite Yersin to Bombay to undertake the treatment of plague. Accordingly, he(7) arrived in March 1897 with a supply of serum and treated some 50 cases in private practice, along with other medical officers, he also treated some cases admitted into hospitals with his serum. The other sera which had a trial are those of Terni (Messina), of Brazil (Brazil), of Tavel (Berne), of Paulthaus (Vienna), of Shibayama (Japan), and of Rowland (London).

#### (B) RESULTS OF SERUM THERAPY IN MAN

From the reports of Bannerman(8), Choksy(9), and the Advisory Committee on Plague Investigations in India(10) and of Kolmer(11) and other recent publications, we have summarized in Tables I and II the results which have hitherto attended the use of anti-plague sera not only in our plague hospitals but also in countries outside India. From these it would appear that among the class of patients who seek admission into our hospitals, the administration of serum was attended with a reduction in the case mortality by about 7 to 10 per cent, whereas the case mortality following its use in other countries has varied within very wide limits, namely, from 5.5 to 75.0 per cent in different epidemics. To account for these differences it is, therefore, necessary to consider the unfavourable factors which have attended its use in our hospitals in Bombay, in some detail. Some of these are —



TABLE II  
*Serum therapy of bubonic plague (outside India)*

1896-1912. KOLNER (1925) INFECTION IMMUNITY AND BIOLOGIC THERAPY					1912-1927 FURTHER INFORMATION PUBLICATIONS						
Author	Epidemic	Year	Cases treated	Percentage mortality	Serum used	Author	Epidemic	Year	Cases treated	Percentage mortality	Serum used
Yersin	China	1896-98	79	27.1	Pasteur Institute	Loidl	China	1912	53	56.6	Pasteur Institute
Zabolotny	Mongolia	1898	16	75.00		Doucet	New Caledonia	1912		Very useful	
Delay	Mongtze	1898	10	60.00		Hofstadter	Amman	1908-13	195	65.6	
Throux	Tamatave, Madagascar	1898-99	20	55.00		Seeman	New Orleans	1915	18	16.6	
Calmette, Salubert and Metin	Oporto, Portugal	1899	118	11.8	Pasteur Institute	Manziola and Broca		1918	21	23.8	
Godinho	Santos Brazil	1900	19	16.8		Levy Dru		1920	36	47.2	
Prumet, Noc	Noumea, Oceania	1899-01	24	11.6		McNicken	Tamatave Madagascar	1921		Satisfactory results	Pasteur Institute
Anber Vassal	Reunions, Indian Ocean	1899-01	21	14.3	Pasteur Institute	Colbert	Tunisien China	1920-21	16	6.2	
Clarac, Mongay	Majunga Madagascar	1902	71	15.0		Armstrong	Sidney	1921	11	9.0	
Roufflands	China	1902-03	168	50.6		Lavandro	Porto Rico	1921		Great value	
Agote and Medina	Rozario, Brazil		26	12.3		Zabolotny	S. Russia and Manchuria	1922		Very useful	
Del Rio and Zegers	Imque	1903	85	14.7		Joltrain and de Genies	Paris	1922		Greatly impressed	
Montero	Autofogasta		50	6.0		Heggs	Bagdad	1922-23	Completely justifies its use		Pasteur Institute
Cruzat	Chanaru	1904	18	5.5		Andruzzi	Italian Somali land	1923-24		16.3	
Penna	Buenos Aires, Argentine	1905	201	19.3	Pasteur Institute	Chunmays Vysishka	Turkistan	1924	16	68.7	
Duprat, Taveres de Marceide	Rio Janeiro, Brazil	1900-06	2,572	25.5		Cumpston	Australia	1900-25		Some value	
Ferrari	Do	1907	69	7.2		Asice	Madagascar	1924-26	19	26.3	
Burnett	Queensland	1900-07		29.7		Dawson	Burma	1927	50	32.0	Pasteur Institute
Penna	Argentina	1905-12		12.5		Adier	Emyrno, Madagascar	1927	33	39.4	
Do	Do	1914-19		7.8							

(a) *The virulence of epidemics*

From Table III, it would appear that although the incidence of plague in Bombay has been gradually on the decline, yet the virulence of infection, judged by the case mortality either among the cases reported in the city or among the patients admitted into our plague hospitals, has remained the same throughout the successive epidemics

TABLE III

*The incidence and case mortality of bubonic plague in the city of Bombay and in the plague hospitals*

Population	BOMBAY				PLAGUE HOSPITALS		
	Year	Attacks	Deaths	Percentage mortality	Admissions	Deaths	Percentage mortality
821,764	1896	2,544	1,936	76.1	297	199	67.0
	1897	13,314	11,003	82.6	1,270	862	67.8
	1898	22,130	18,185	82.1	2,187	1,602	73.2
	1899	19,551	15,796	80.7	1,387	1,088	78.4
	1900	17,913	13,285	74.1	812	609	75.0
776,006	1901	21,006	18,736	89.1	1,518	1,168	76.9
	1902	16,423	13,820	84.1	1,258	1,026	81.5
	1903	23,344	20,788	89.0	1,371	1,046	76.2
	1904	15,488	13,538	87.4	1,080	797	73.8
	1905	16,308	14,198	87.0	1,395	1,051	75.3
	1906	12,323	10,823	87.8	944	666	69.4
	1907	7,353	6,389	86.8	611	445	72.8
	1908	6,134	5,361	87.3	609	432	70.9
	1909	5,864	5,197	88.6	608	488	80.2
	1910	4,130	3,656	88.5	440	339	77.0
979,445	1911	4,503	4,006	89.9	565	423	74.8
	1912	1,936	1,717	88.6	238	174	73.1
	1913	3,023	2,609	86.3	392	293	74.7
	1914	3,348	2,941	87.8	440	332	75.4
	1915	694	599	86.3	135	97	71.8
	1916	2,321	1,987	85.6	411	319	77.6
	1917	1,987	1,706	85.8	476	359	75.4
	1918	1,380	1,143	82.8	328	263	80.1
	1919	826	702	84.9	189	145	76.7
	1920	347	282	81.2	123	79	64.2
1,175,914	1921	1,031	811	78.6	339	262	77.2
	1922	759	632	83.6	234	180	76.9
	1923	1,501	1,329	88.5	343	264	76.9
	1924	450	409	90.8	120	97	80.8
	1925	192	174	90.6	56	48	85.7
	1926	63	56	88.8	25	22	88.0
	1927	223	207	92.8	61	57	93.4
	1928	296	257	86.8	129	109	84.0

(b) *The natural resistance to infection*

The patients who are admitted into our hospitals are mainly from the very poorest classes of the community they are ill-nourished, they live in over-crowded dwellings under insanitary and unhygienic surroundings, moreover, the different races such as the Hindus, Mahomedans, Native Christians, Parsees and Jews, admitted into hospital differ in their natural resistance to infection, the Hindus who form the largest number being the most susceptible. In Table IV, we have recorded some of Choksy's figures on the racial incidence of plague and its fatality among cases admitted to Arthur Road and Maratha Plague Hospitals.

TABLE IV

*Racial incidence of plague and its fatality among cases admitted to Arthur Road and Maratha Plague Hospitals, Bombay*

Races	SEPTEMBER 1896 TO JUNE 1900		1902 TO 1905		1896 TO 1918	
	Cases admitted	Percentage mortality	Cases admitted	Percentage mortality	Cases admitted	Percentage mortality
Hindus	5,018	71.67	1,880	79.71	13,228	77.2
Mahomedans	556	65.73	770	73.50	2,335	69.3
Native Christians	460	64.34	399	65.91	1,676	68.1
Parsees	36	58.33	15	40.00	305	56.0
Jews	21	66.66	10	80.00	56	64.3
Europeans and Anglo Indians					68	55.8

(c) *The duration of illness*

In the absence of compulsory isolation, most of the patients only seek admission into hospitals when treatment in their own homes has failed, or when they are abandoned by their relatives and friends. In plague, which normally proves fatal in three to six days, absence of proper treatment during the first day or two of illness must seriously influence the case mortality. According to Choksy(12) fully 70 per cent of the patients are admitted into our hospitals with septicaemia, the average duration of illness on admission being 3 to 4 days. The mortality among hospital patients within 48 hours of admission has been shown by Choksy(13) to vary between 57 and 62 per cent. Table V compiled from the results of Yersin(14) and of Choksy(15) emphasizes the importance of early treatment with serum.



TABLE V

## Serum therapy of bubonic plague

## Results of treatment according to the duration of illness

Duration of illness	YERSIN (1896) ANJOY			YERSIN (1897) BOMBAY			CHOKSY (1908) BOMBAY					
	PASTEUR INSTITUTE SERUM			PASTEUR INSTITUTE SERUM			LUSTIG'S SERUM		IN HOSPITAL CASES		IN PRIVATE PRACTICE	
	Cases treated	Percentage case mortality		Cases treated	Percentage case mortality		Cases treated	Percentage case mortality	Cases treated	Percentage case mortality	Cases treated	Percentage case mortality
1st day of illness	6	0 00		17	12 00		316	30 3	37	45 9	116	28 4
2nd "	10	0 00		17	35 00		300	52 6	138	61 5	77	42 8
3rd "	4	0 00		12	50 00		216	63 0	145	66 8	37	67 5
4th "	2	50 00		3	67 00		105	57 1	72	56 9	5	40 0
5th "	1	100 00		1	100 00		52	61 5	36	58 3	7	71 4
6th "							14	57 1	7	57 1	1	100 0
7th "							4	100 0	3	100 0	1	100 0
TOTAL	23	8 7		50	34 00		1037	49 2	438	61 1	244	40 7

*(d) The therapeutic dose of serum*

The available records of cases treated with serum in India during the years 1897—1912 seem to indicate that the amount of serum administered was relatively small and the injections were mostly given by the subcutaneous method. Kolmer (16) has pointed out that 'the prompt administration of anti-plague serum in large doses and by intravenous injection has proved of decided value especially in the treatment of the bubonic type of the disease in the highly fatal pneumonic type it has apparently saved some lives.'

*(e) The estimation of the curative value of serum*

Choksy has been contending against the 'alternate system' employed by Haffkine and the Plague Commission for estimating the curative value of the anti-plague sera in our hospitals. This system consists in subjecting every second arrival into the hospital with the exception of those who died within a few hours of admission to injections with serum, without selecting the patients for treatment according to personal impressions or statements supplied by the medical officer or by the patient or his relatives and comparing the mortality statistics among the treated and among the non-treated, to estimate the curative value. On the other hand, Choksy exercised a 'system of selection' for treatment by excluding all moribund cases and those who were in a semi convalescent or convalescent condition at the time of admission and treated only such cases who seemed to him would be benefited by the serum. The results which have attended these two methods show that with Yersin's serum by the 'alternate system' there was a reduction in the case mortality by 6.8 per cent as compared with the 'system of selection' which was attended with a reduction in the case mortality by 23.2 per cent, and in the case of Lustig's serum the 'alternate system' showed a reduction of 7.4 per cent as compared with the 'system of selection' which showed a reduction of 21.7 per cent.

Clinical observations which now extend over thirty years have shown that following the injections of serum there is a moderation in the intensity and duration of fever, improvement in the state of circulation as indicated by increase of arterial pressure, diminution in the size of, and lessening of pain in buboes, cessation in the progress of advancing lymphatic infection clearing of the mental faculties and a general improvement in the condition of the patient. Even in cases where the administration of serum fails to save the life of the patient, it produces great amelioration in his condition and prolongs life. These clinical phenomena which manifest themselves after the injection of serum have made the physicians hope that with a serum of greater potency and of anti-toxic property, it will be possible to save more lives and to advocate for further researches into the anti-plague serum.

**(C) OUR ATTEMPTS AT THE PREPARATION OF POTENT SERUM**

Since the discovery of anti-diphtheria serum, considerable advances in our knowledge of bacteriology and immunology have been made and we have come to

recognize many factors which may have an influence on the potency of anti-sera. Some of these are —

(a) *The strain of the organism used for immunization*

For the preparation of a potent anti-bacterial serum, the first requisite is the selection of a suitable strain or strains of an organism, just as the employment of a powerful toxin is essential for the preparation of a potent anti-toxic serum. Many attempts have been made to differentiate the strains of bacilli isolated in different countries by the agglutination and absorption tests, by the carbohydrate reactions and by complement fixation tests but without success. D'Herelle (17) believes that strains of plague bacilli isolated in India, are much more virulent than those isolated in other countries and the Indian strain has been shown to be relatively more resistant to his bacteriophage isolated in Indo-China and in Alexandria. Some investigators hold that the plague bacillus produces an exotoxin in broth cultures and Markl (18) has employed filtrates of broth cultures for the immunization of animals to obtain a potent anti-plague serum. Although the occurrence of giant colonies in cultures of plague has long ago been observed by Haffkine (19), yet, it is only recently that Arkwright (20) has drawn attention to the difference in the antigenic properties of the rough and smooth strains of the colityphoid group of organisms. Further researches have shown that this difference in the antigenic value between the rough and the smooth strains holds true with different groups of organisms hitherto examined. Huntoon and Hutchinson (21) have pointed out that in the preparation of anti-plague serum 'owing to the dangers connected with handling the plague organism, commercial laboratories are required to use only non-virulent strains'. Our experiments with anti-plague vaccines have shown that (i) the immunizing value of a vaccine is largely dependent on the virulence of the strain employed for its preparation, (ii) strains isolated either from human cases or from experimentally infected rats, exhibit individual variations in virulence, (iii) emulsions made from plague-infected spleen are more virulent than those obtained from cultures on agar or in broth, (iv) virulence of the strain is lost wholly or in part under prolonged cultivation on artificial media, (v) virulence of the organism is modified by passages from a highly susceptible animal into animals of relatively low susceptibility, and (vi) an avirulent strain produces a vaccine of low potency. Therefore, for the immunization of animals, we have uniformly employed strains of great virulence recently isolated from human cases and whose virulence has been maintained by repeated passages in the highly susceptible Madras rats. Cultures were made on agar and after an incubation of 48 hours at room temperature, the growth was washed with the supernatant fluid of Haffkine's plague prophylactic vaccine and used for inoculation of the animals.

(b) *Selection of a suitable animal for immunization*

Since the preparation of diphtheria anti-toxin in horses, investigators have as a rule employed horses for the preparation of other anti-sera. While the therapeutic value of anti-diphtheria, anti-tetanus and anti-streptococcus sera is now well

established the success which has attended the use of other sera has been limited. In the case of diphtheria, tetanus and streptococcal infections, it has long been recognized that these diseases occur in horses under natural conditions. For the production of anti-plague serum Yersin and Roux, Kolle, Lustig and others have employed horses for immunization and the therapeutic results which have attended its use have varied with the potency of different sera and with the virulence of the epidemics in different countries. Haffkine (22) during the course of his experiments on the production of anti-plague serum found that sheep yielded a serum which was therapeutically more promising than those obtained from other animals including horses. Term (23) in Messina found that the anti-plague serum prepared by the immunization of mules and cattle gave better results than that obtained from horses. It has also been recorded that during epidemics of plague in Europe and in China there had been an unusually heavy mortality among cattle, sheep and pigs, but no mention is made of such an occurrence in horses. Furthermore haemorrhagic septicaemia, a disease caused by the members of the *Pasteurella* group, is a common and fatal infection in cattle, sheep and pigs but not in horses. To what extent the susceptibility of an animal to infection by a particular organism, has an influence in the production of a potent anti serum specific for that organism is a subject which seems to have received little attention. Therefore in the first instance, we have carried out experiments to study the effects of hyper-immunization with virulent cultures in rabbits, animals highly susceptible to experimental infection with plague and to estimate the potency of the immune rabbit serum in experimentally infected rabbits. The results of these experiments which are detailed in Tables VI to XVII showed that (a) rabbits withstand repeated injections with virulent cultures at weekly intervals over a prolonged period of time, namely, two years (b) during this long course of treatment with cultures, most of the animals gain weight, while some develop joint affections and in a few cases paralysis of the hind limbs occurs, (c) animals which die during the course of immunization do not show on post-mortem examination the presence of plague bacilli either in their blood or in the internal organs, although signs of recovered-plague are met with in some of the rabbits, (d) rabbits immunized by the subcutaneous route yield sera of relatively low agglutinating titre as compared with those immunized by the intravenous route, (e) the therapeutic results which attend the use of sera of relatively low agglutinating titre are less marked compared with those following the use of sera of high titre, (f) when the serum is administered intravenously in a single dose of 1 to 2 c.c. after 48 to 72 hours of infection, the mortality among the infected rabbits is negligible, and (g) the agglutinating titre of the serum used for therapeutic use is possibly one of the factors which determine the potency of the serum. From these results it would appear that, for therapeutic use, a potent serum can be prepared in a susceptible animal. But, in view of the fact that only very small amounts of sera can be obtained from immunized rabbits, the production of anti-plague serum from these animals can have no practical application in the treatment of human plague.



[illegible]

Note —I = Intravenously, S = Subcutaneously \* = serum of comparatively low agglutinating titre (1 1600 to 1 4000)

*Result*—Not a single animal died, when the treatment with serum was commenced within a few minutes of infection. A dose of 2 c.c. intravenously along with 5 c.c. subcutaneously seemed enough to save the life of the animal, repeated doses of serum and in quantities given had no adverse effect on the life of the animal. These animals were under observation for a period of 30 days from the date of infection, and all survived.



1670	21	20	20	21	20	20
1000	21	20	21	20	20	20
1670	20	20	21	20	20	20
1980	20	20	21	20	20	20
1850	21	20	21	20	20	20
1900	21	20	21	20	20	20
1750	22	20	21	20	20	20
2000	21	20				
1700	21	20				
2150	*20	20				
1550	*21	20				
1820	21	20				
1670	21	20				

Note.—I = Intravenously S = Subcutaneously \* = serum of comparatively low agglutinating titre

**Note** —I = Intravenously S = Subcutaneously \* = serum of comparatively low agglutinating titer

**Result** —Not a single animal died under treatment when it was commenced after 24 hours of infection. A single dose of 2 c.c. administered intravenously was sufficient to save the life of the animal, repeated doses had no adverse effect. These animals were under observation for a period of 30 days after infection, and no deaths occurred among them during this interval.



TABLE VIII

*Treatment in rabbits experimentally infected with plague with serum prepared by immunization of rabbits*  
(Results of seven experiments carried out between 14-2-1927 and 14-10-1927 )

FIRST SERIES

Rabbits were infected with our test dose and treatment with serum was commenced after 48 hours of infection ,  
serum treatment was repeated at intervals of 24 hours

Rabbit's weight in grammes	1	2		3		4	5	6	7	8	9	10	11	12	13	14	15
	I S	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S
1720		2	0	2	0	2	0	2	0	2	0						
1600		2	0	2	0	2	0	2	0	2	0						
2000		*2	0	2	0	2	0	2	0	2	0						
1750		*2	0	2	0	2	0	2	0	2	0						
1900		2	0	2	0	2	0	2	0	2	0						
1800		2	0	2	0	2	0	2	0	2	0						
1900		2	0	2	0	2	0	2	0	2	0						
1710		2	0	2	0	2	0	2	0	2	0						
2000		2	0	2	0	2	0	2	0	2	0						
1880		2	0	2	0	2	0	2	0	2	0						
1095		*1	5	1	5	1	5	2	0	2	0						
1700		*1	5	1	5	1	5	2	0	2	0						

1700	2	0	2	0	2	0	2	0	2	0										
1850	2	0	2	0	2	0	2	0	2	0										
2000	2	0	2	0	2	0	2	0	2	0										
1730	2	0	2	0	2	0	2	0	2	0										
1650	2	0	2	0	2	0	2	0	2	0										
2050	*2	0	2	0	2	0	2	0	2	0										
1700	*2	0	2	0	2	0	2	0	2	0										
1900	2	0	2	0	2	0	2	0	2	0										
1800	2	0	2	0	2	0	2	0	2	0										
1000	2	0	2	0	2	0	2	0	2	0										
1050	2	0	2	0	2	0	2	0	2	0										
1880	2	0	2	0	2	0	2	0	2	0										
1650	2	0	2	0	2	0	2	0	2	0										

*Note* —I = Intravenously S = Subcutaneously D = Died and when its spleen was cutaneously infected into a rat it died of plague, d = died, but when its spleen was cutaneously infected into a rat it did not die for a period of 15 days

*Result* —Out of the 25 rabbits in whom the treatment with serum was commenced after 18 hours of infection, 1 died within ten days of infection, out of the ten rabbits treated with a single administration of serum intravenously in a dose of 2 cc two died with a case mortality of 20 per cent, but the case mortality among all the animals was 16 per cent, when the treatment was repeated at intervals of 24 hours.

\* = serum of comparatively low agglutinating titre

TABLE IX

*Treatment in rabbits experimentally infected with plague with serum prepared by immunization of rabbits*  
*(Results of seven experiments carried out between 14-2-1927 and 14-10-1927)*

## FIRST SERIES

Rabbits were infected with our test dose and treatment with serum was commenced after 72 hours of infection, serum treatment was repeated at intervals of 24 hours

Rabbit's weight in grammes	1		2		3		4		5		6		7		8		9		10		11		12		13		14		15	
	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S
1500					2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0								
1820					2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0								
1720					2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0								
1590					2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0								
1800					2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0								
1800					2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0								
1900					2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0								
1750					2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0								
1920					2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0								
1750					2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0								
1900					2 0	4 0	4 0	4 0	4 0	4 0	2 0	2 0	2 0	2 0	2 0	d	2 0	2 0	2 0	2 0	2 0	2 0								
1630					*1 5	1 5	1 5	1 5	1 5	1 5	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0								

1650	2	0	2	0	0	0	0	2	0										
1780	2	0	2	0	2	0													
1950	*2	0	2	0	1	0	1												
2000	*1	2	1	2	1	2	1												
1700	2	0	1	0	1														
1920	2	0	2	0															
1950	*2	0	2	0	d														
1800	*2	0	2	0	d														
1720	2	0																	
1600	2	0																	
1900	2	0																	
1800	2	0	d																
1950	2	0																	
1710	2	0																	
1970	2	0																	
1620	2	0																	
2000	*2	0	d																
1800	*2	0																	

Note — I = Intravenously, S = Subcutaneously, D = Died and when the spleen is passaged into rat cutaneously, it died of plague, d = died, but the rat when passaged with the spleen did not die in 15 days from the time of passage

Result — Out of the ten rabbits which received a single dose of the serum intravenously of 2 cc, 3 died with a case mortality of 30 per cent while the mortality among all the rabbits treated at the end of 72 hours with one or more doses was also 30 per cent. The survivors were under observation for a period of 30 days from the date of infection and none succumbed during that interval. \* = serum of comparatively low agglutinating titre





TABLE XI.

*Treatment in rabbits experimentally infected with plague with serum prepared by immunization of rabbits.*  
*(Results of seven experiments carried out between 14-2-1927 and 14-10-1927)*

## FIRST SERIES

Rabbits were infected with our test dose and treatment with serum was commenced after 120 hours of infection, serum treatment was repeated at intervals of 24 hours

Rabbit's weight in grammes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	I S	I S	I S	I S	I S	I S	I S	I S	I S	I S	I S	I S	I S	I S	I S
1500						2 0	2 0	2 0	2 0	2 0	2 0				
1700						2 0	2 0	2 0	2 0	2 0	2 0				
1520						2 0	2 0	2 0	2 0	2 0	2 0				
1950						4 5	4 5	4 0	4 0	2 0	2 0				
2000						d									
1600						D									
1700															
1530															
1750						d									
1680						2 0	2 0	2 0	2 0	2 0	2 0	2 0			
1680						2 0	D								
1680						2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0		
1900						2 0	2 0	2 0	2 0	2 0	2 0	2 0	2 0		
1680							2 0	2 0	2 0	2 0	2 0				

Note — I = Intravenously S = Subcutaneously \* = serum of comparatively low agglutinating titre D = Died and when the spleen was passed into a rat it also died of plague, d = died, but the passage rat survived for 15 days from the date of passage

Results — Even when the treatment was commenced after 120 hours and 144 hours of infection a single dose of 2 c.c. of serum of high agglutinating titre protected the animal from death These treated animals were under observation for a period of 30 days from the date of infection and there was no mortality among the survivors





TABLE XIII

*Treatment in rabbits experimentally infected with plague with serum prepared by immunization of rabbits*  
*(Results of three experiments carried out between 23-1-1928 and 14-4-1928)*

## SECOND SERIES

Rabbits were infected with our test dose and treatment commenced within a few minutes of infection, serum treatment was by the intravenous route and was repeated at intervals of 24 hours

Rabbit's weight in grammes	Same time	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1900	1	1	1	1	1	1	1	1	1							
1600	1	1	1	1	1	1	1	1	1							
1900	1	1	1	1	1	1	1	1	1							
1700	1	1	1	1	1	1	1	1	1							
1900	1	1	1	1	1	1	1	1	1							
1650	1	1	1	1	1	1	1	1	1							
1800	1	1	1	1	1	1	1	1	1							
1700	1	1	1	1	1	1	1	1	1							
1850	1	1	1	1	1	1	1	1	1							
1650	1	1	1	1	1	1	1	1	1							
1950	1	1	1	1	1	1	1	1	1							
1650	1	1	1	1	1	1	1	1	1							
1900	1	1	1	1	1	1	1	1	1							
1700	1	1	1	1	1	1	1	1	1							
1900	1	1	1	1	1	1	1	1	1							
1700	1	1	1	1	1	1	1	1	1							
1800	1	1	1	1	1	1	1	1	1							
1750	1	1	1	1	1	1	1	1	1							

*Result*—All the animals survived the infection even when the dose of serum was only 1 c.c given intravenously. These animals were under observation for a period of 30 days and none died during this period.

TABLE XIV.

*Treatment in rabbits experimentally infected with plague with serum prepared by immunization of rabbits*  
*(Results of three experiments carried out between 23-1-1928 and 11-1-1928)*

## SECOND SERIES.

Rabbits were infected with our test dose and treatment was commenced after 24 hours of infection, serum treatment was repeated at intervals of 24 hours, serum injections were made intravenously

Rabbit's weight in grammes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1950	1	1	1	1	1	1	1	1	1						
1700	1	1	1	1	1	1	1	1	1						
1900	1	1	1	1	1	1	1	1	1						
1750	1	1	1	1	1	1	1	1	1						
1920	1	1	1	1	1	1	1	1	1						
1800	1	1	1	1	1	1	1	1	1						
1950	1	1	1	1	1	1	1	1	1						
1800	1	1	1	1	1	1	1	1	1						
1920	1	1	1	1	1	1	1	1	1						
1920	1	1	1	1	1	1	1	1	1						
2000	1	1	1	1	1	1	1	1	1						
1750	1	1	1	1	1	1	1	1	1						
1950	1	1	1	1	1	1	1	1	1						
1800	1	1	1	1	1	1	1	1	1						
1900	1	1	1	1	1	1	1	1	1						
1750	1	1	1	1	1	1	1	1	1						
1900	1	1	1	1	1	1	1	1	1						
1700	1	1	1	1	1	1	1	1	1						

*Result* —All the animals survived the infection even with a single dose of serum of 1 c.c. given intravenously. These animals were under observation for a period of 60 days without any deaths during this interval.

TABLE XV

*Treatment in rabbits experimentally infected with plague with serum prepared by immunization of rabbits*  
*(Results of three experiments carried out between 23-1-1928 and 14-4-1928)*

## SECOND SERIES

Rabbits were infected with our test dose and treatment was commenced after 48 hours of infection, serum treatment was repeated at intervals of 24 hours, serum injections were made intravenously

Rabbit's weight in grammes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1550		1	1	1	1	1	1	1	1	1					
1550		2	2	2	2	2	2	2	2	2					
1580		1	1	1	1	1	1	1	1	1					
1600		2	2	2	2	2	2	2	2	2					
1680		1	1	1	1	1	1	1	1	1					
1670		2	2	2	2	2	2	2	2	2					
1670		1	1	1	1	1	1	1	1	1					
1670		2	2	2	2	2	2	2	2	2					
1700		1	1	1	1	1	1	1	1	1					
1750		2	2	2	2	2	2	2	2	2					
1770		1	1	1	1	1	1	1	1	1					
1770		2	2	2	2	2	2	2	2	2					
1770		1	1	1	1	1	1	1	1	1					
1720		2	2	2	2	2	2	2	2	2					
1720		1	1	1	1	1	1	1	1	1					
1800		2	2	2	2	2	2	2	2	2					
1820		1	1	1	1	1	1	1	1	1					
1840		2	2	2	2	2	2	2	2	2					

*Result* —All the animals survived the infection, a single dose of 1 c.c. injected intravenously seemed sufficient to protect the animals from death, these animals were under observation for a period of 60 days and none died during this interval

*Treatment in rabbits experimentally infected with plague with serum prepared by immunization of rabbits*  
(Results of three experiments carried out between 23-1-1928 and 14-4-1928)

B. P. B. Naidu S. Tung and K. H. Kamaoka

SECOND SERIES

Rabbits were infected with our test dose and treatment was commenced after 72 hours of infection, serum treatment was by the intravenous route and was repeated at intervals of 24 hours

Rabbit's weight in grammes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1550			1	1	1	1	1	1							
1550			2	2	2	2	2	2	2	2					
1550			1	1	1	1	1	1	1	1					
1620			2	2	2	2	2	2	2	2					
1620			1	1	1	1	1	1	1	1					
1670			2	2	2	2	2	2	2	2					
1680			1	1	1	1	1	1	1	1					
1690			2	2	2	2	2	2	2	2					
1720			1	1	1	1	1	1	1	1					
1730			2	2	2	2	2	2	2	2					
1730			1	1	1	1	1	1	1	1					
1780			2	2	2	2	2	2	2	2					
1780			1	1	1	1	1	1	1	1					
1770			2	2	2	2	2	2	2	2					
1810			1	1	1	1	1	1	1	1					
1810			2	2	2	2	2	2	2	2					
1810			1	1	1	1	1	1	1	1					
1840			2	2	2	2	2	2	2	2					

*Result* — Out of the 18 animals in whom treatment was commenced after 72 hours of infection, six died with a mortality percentage of 33.3, as to the shortage of serum both the highly agglutinating serum and one of comparatively low agglutinating titre were employed

D\* — This animal died of complications The survivors were under observation for 60 days after infection without any further deaths

TABLE XX

*Rats treated by subcutaneous injection of anti-plague serum after 24 hours of experimental infection with plague.*

THERAPEUTIC DOSE OF SERUM = 1 c c

Dates of Experiments	Rats used	Sheep No 14 Serum Agglutination 1-64														
		Daily mortality														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
10-6-29	25	0	2	1	1	4	6	3	2	1	0	4	0	0	1	0
21-6-29	25	0	0	0	2	1	2	3	1	3	3	0	0	2	2	1
10-7-29	25	0	0	0	6	1	2	2	2	2	3	0	0	0	2	1
19-7-29	25	0	0	0	0	1	5	1	0	0	7	1	4	3	1	0

Mortality	43 per cent	Total mortality	89 per cent
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Dates of Experiments	Rats used	Pasteur Institute Serum Agglutination Nil														
		Daily mortality														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
10-6-29	25	0	1	2	11	5	4	0	1	0	0	0	0	0	0	0
21-6-29	25	0	0	4	10	2	0	3	1	2	0	1	0	0	0	0
10-7-29	25	0	0	3	9	3	2	4	0	2	1	0	0	0	0	0
19-7-29	25	0	1	1	3	9	1	5	1	0	0	1	0	0	0	0

Mortality	83 per cent	Total mortality	93 per cent.
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Dates of Experiments	Rats used	Controls														
		Daily mortality														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
10-6-29	25	0	0	7	10	3	1	0	0	0	0	0	1	0	0	0
21-6-29	25	0	0	12	5	5	0	0	2	0	0	0	0	0	0	0
10-7-29	25	0	0	12	9	2	0	0	0	0	0	0	0	0	0	0
19-7-29	25	0	0	8	11	4	0	1	0	0	1	0	0	0	0	0

Mortality	90 per cent	Total mortality	94 per cent
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## IMMUNIZATION OF SHEEP AND CALVES

We have already referred to some of the considerations which led us to hope for the production of a potent serum in sheep and calves. For this purpose we began with the immunization of sheep with virulent cultures, the injections were all given intravenously and at weekly intervals, we began with very small doses of live culture namely 0.25 c.c. of a broth culture of 18 hours or with one-twentieth of an agar slope of 18 hours growth and very gradually increased the doses until we reached a dose of 50 agar cultures for an injection. We also examined the agglutinating titre of the serum of animals under immunization at intervals of 10 to 12 weeks. Before the commencement of injections, the sera were tested for their agglutination and none of them had any agglutinating property even in a dilution of one in two. Contrary to our expectation, we lost a very large number of animals during the course of immunization, for, out of a total of 21 sheep 1 died within 8 weeks of the commencement of injections, one within 5 months, 3 within 7 months and 2 within 11 months. During the course of immunization the weekly injections had often to be put off because of the illness and disabled condition of the animals. Following on each injection there was a rise of temperature which lasted from 24 to 48 hours, during this period the animal was dull and off its feed, in some this was followed by diarrhoea and joint swellings. All the animals under treatment began to lose weight. By April 1929 we had only three animals left, of these, one received in all 50 injections, another 11 injections and the third 15 injections. Following on the repeated injections of cultures the agglutinating titre of the serum gradually rose between 1 in 64 and 1 in 128 (water clear). We now bled the animals and estimated their therapeutic value in experimentally infected Madras rats along with Pasteur Institute's anti-plague serum for comparison with adequate controls. The results are detailed in Tables XVIII to XXII, from these it would appear that the serum prepared from sheep is more potent than that of the Pasteur Institute, the difference in the mortality rate following the use of the two sera is brought out clearly at the end of the first week of infection by which time almost all the controls have succumbed. But, in view of the very heavy mortality among sheep during the course of immunization, we next proceeded to immunize calves and to study the potency of calf's serum in experimentally infected animals.

Before undertaking the hyper-immunization with virulent cultures it is necessary, in the first instance, to study the results of infection in calves both by the subcutaneous and by the intravenous route. Haemorrhagic septicaemia in bovines is usually an acute and generalized infection attended with high mortality whereas bovine lymphangitis is but a chronic and localized infection of the lymphatic glands and is rarely fatal. In both these diseases, the causative organisms are members of the *Pasteurella* group closely allied to *B. pestis* in their morphological and cultural characters. In bovine lymphangitis, Sheather (29) reproduced identical lesions in hill bulls and plains calves by the subcutaneous injection of the pus or



organisms isolated from the pus from cases of this disease Krishnamurti (30) not only confirmed the results of Sheather but in a personal communication claims that his experiments have established the close relationship of the organism of bovine lymphangitis with that of *B. pestis*. We carried out a comparative experiment in two calves, 18 months old and weighing about 300 lbs., by injecting virulent cultures of plague, both by the subcutaneous and by the intravenous route. The subcutaneous injection of two agar slopes on the right side of the neck produced in one animal a rise of temperature to  $105.5^{\circ}\text{F}$  within 24 hours of infection, attended with a painful swelling measuring 4" by 2" along with enlargement of the prescapular gland, the swelling gradually increased in size and by the eighth day it resulted in an open sore, on the fourteenth day, when the swelling became considerably less and the sore began to heal, we repeated the subcutaneous injection of eight agar slopes on the left side of the neck, again the temperature rose to  $103.8^{\circ}\text{F}$  (the normal being  $100^{\circ}$  to  $101^{\circ}\text{F}$ ) attended with a swelling measuring 8" by 4" and enlargement of the prescapular gland on that side and the animal was off its feed for 2 days following the injection. The other animal was given an intravenous injection of 0.2 of an agar slope, the injection was followed by a rise of temperature to  $105^{\circ}\text{F}$  within 48 hours of infection which persisted as an irregular fever with a maximum temperature of  $106.8^{\circ}\text{F}$  on the 4th and 5th days of inoculation followed by a gradual fall to normal. A second dose of 0.2 of an agar slope was given intravenously on the 8th day, this dose did not seem to produce any marked reaction and the animal seemed bright, a third injection of 0.5 of an agar slope gave rise to a temperature of  $105^{\circ}\text{F}$  at the end of 24 hours which fell to normal after 48 hours of fever without any visible ill-effects. We then proceeded to immunize these two animals at weekly intervals with increasing doses of the culture. In the case of the first animal, the infecting dose was rapidly increased, beginning with 10 agar slopes, each subsequent dose was increased by five agar slopes, and in the other animal the doses were increased at first by one agar slope only. In the first animal the reaction following each injection was pronounced and the temperature persisted for 48 hours before reaching normal. In the second animal the injections were attended with only a slight rise of temperature, namely, one degree above normal, and this lasted for a period of 12 hours. The first animal following on its 7th injection of a dose of 40 agar slopes showed very marked reaction attended with signs of dyspnoea lasting for a short time. After this, following every injection of 40 or 50 agar slopes there was a rise of temperature between  $105^{\circ}$  and  $106^{\circ}\text{F}$  within 12 hours of injection and lasting for 36 hours with increasing dyspnoea, and on the night of the 11th intravenous injection with a dose of 50 agar slopes, the animal collapsed and died struggling for breath. Post-mortem examination showed that in the region of the neck where the injection was made, there was a blood-stained gelatinous oedema which extended to both sides of the neck, there was also infiltration of tissues surrounding the veins. The right prescapular gland was enlarged to the size of a walnut and on section it showed a thick capsule enclosing pus without any glandular material. On opening the thorax, there was a large amount

TABLE XXIII

*Susceptibility of rabbits to Experimental Infection with our Test Dose, namely,  
0.003 mg of Spleen of a rat died of Plague*

Year	No of Experiments	Weight in grammes	Days after Infection														
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1926	3	2350			D												
		2470					d										
		2100					D										
		*2550															
		2100					D										
		2130			D												
		2480			D												
		*2570															
		1770					D										
		1930			D												
		1790						d									
		*1140															
		1770						d									
		1460							D								
		1650						D									
		*1820															
		1840			d												
		1550					D										
		*1460															
		*1970															
1927	10	2000			D												
		1420			D												
		2080			D												
		1500			D												

*Note*—D = Died and when the spleen was passaged into a rat, it also died of plague  
d = died, but the passage rat survived for 15 days from the date of infection, \* = The animal survived during the 15 days of infection

TABLE XXIII—*conold*

Year	No of Experiments	Weight in grammes	Days after Infection														
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
		1700			D												
		1600			D												
		2080			D												
		1950			D												.
		2560			D												.
		2520			D												
		2000			d												
		2340			D												
		2330			D												
		2130			d												
		1040						d									
		1900				D											
		2030			d												
		1650			D						.						
		1650			D												
		1680			D												.
		1830			D												.
		1650			D												..
		1660				D											..
		1880				D											.
		1610				D											..
		1600				D											
		1570							d								
		1550								d							.
		*1570															..

*Note*—D = Died and when the spleen was passaged into a rat, it also died of plague, d = died, but the passage rat survived for 15 days from the date of infection, \* = The animal survived during the 15 days of infection

### Anti-plaque Serum Treatment Ex.

Treatment commenced after 72

Serum 2 c e administered intravenously

CAT SCRUM															Scrum drawn in	Weigh rabbi <sup>n</sup>	TOTAL	
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15			20	
I	I	I					I	I	D							Junc, 1929	2036	
I		I							I								202	14
I		D															2010	
		I															2010	
I		D															2000	19
I	I	I				D											1970	17
		I	I														1950	
		I	I														1920	15
																	1910	20
I		I															1830	
		I															1800	4
		I	I					I	I							Do	1930	
		I	D														1900	18
		I	I	I													1840	
																	1800	7
																	1780	2
																	1750	
																	1740	
			I	I	I	I	I	I	I	D							1720	14
			I	I	I	I	I	I	I								1720	
			I	I	I	I	I	I	I								1720	3
				I	I	I	I	I	I								1710	11
				I	I	I	I	I	I	I	I	I					1700	
	I	I	I	I	I	I	D									Do	*1870	4
	I	I															1830	7
	I																1770	
					I				I								1750	
																	1750	
I	I	I																







# VARIATIONS IN BLOOD GROUPING

BY

LIEUT-COL W F HARVEY, I.M.S. (*Retd*)

(*From the Central Research Institute, Kasauli, India, and the Royal College of Physicians Laboratory, Edinburgh*)

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A group 1 (Jansky) standard serum which is defined as one agglutinating the erythrocytes of groups 2, 3 and 4 that is of all other groups except its own group, is found, say, not to agglutinate the erythrocytes of a particular individual. It is then judged that this individual is a group 1 individual without further testing by other such non agglutinating group 1 sera. In the ordinary way of testing blood the two standard sera of groups 2 and 3 are used with which then it is only possible for one of the accepted 4 groups to emerge. That the findings are not absolute is indicated by insistence for purposes of blood transfusion upon always testing the blood of the donor against that of the recipient. In the work here set out I began with investigating the possibility of obtaining more than the 4 standard blood groups by testing each individual's serum and erythrocytes against every other. It was not long before 5 distinct groups emerged. One then realized what the lengthiness of the procedure would be if carried to a conclusion with even a small group of individuals especially as the work was to be combined with an estimate of degree of agglutination in each case. Accordingly I restricted myself to the determination of variation in degree of agglutination and the interaction of sera and erythrocytes of 58 individuals with the sera and erythrocytes of the 5 groups already obtained.

**TECHNIQUE**—The time of observation was restricted to 15 minutes and the greatest dilution of serum to 1 in 16. By using such a short interval of time as 15 minutes there was no possibility of mistaking simple sedimentation for agglutination. Agglutination was taken as positive when clumping was evident to naked eye observation. Continuous observation could be carried out and exact times of agglutination noted because the water bath used was of glass and the capillary tubes containing mixtures could be read *in situ*. A change in the grouping here given, particularly with some of the doubtful cases, might have been found with greater duration of testing, but any technique adopted, whether it involves short or long periods of observation, must be arbitrary in character. The results are presented, with the conditioning here laid down. As the technique was not that of agglutination procedure usually carried out, whether for bacteria or for blood grouping, I give its detail step by step—

(1) Take blood from the individuals under test with a pipette on the day before application of the test. About 2 c.c. blood can easily be collected by bandaging, constricting, massaging and rebandaging



one or more fingers (2) Add some of this blood from the first taken up portion to a watch glass from which an assistant takes up 200 c mm with teated graduated capillary pipette and adds it to 2.8 c c 1.5 per cent citrated normal salt solution, to make a 5 per cent test erythrocyte suspension (3) Place the remainder of the blood as it is obtained, in a small test tube, kept nearly but not quite horizontal so that the blood is distributed in a sloped layer (4) Allow the blood to clot firmly in this sloped position and gain firm attachment to the glass (5) Set the small test tube up vertically when the blood is firmly attached (6) Place these test tubes and the citrated erythrocyte suspension in the ice chest, to remain overnight (7) Have in readiness, next day, for the test a glass water bath at 37°C, vaccine capillary tubes, plasticine, white porcelain slabs with depressions, teated capillary pipettes, watch glasses and an electric bulb behind the water bath to throw a strong beam into it (8) Place one drop of normal salt solution from a teated pipette into 3 successive depressions of a porcelain slab, leaving the first depression without any salt solution (9) Reject the remainder of the salt solution in the pipette and get rid of residual fluid by compressing and relaxing the teat, while the end of the pipette is pressed upon filter paper (10) Take up, with the same pipette, a quantum of testing serum from one of the tubes of blood to be tested (11) Let fall one drop of serum into No. 1 depression, containing no salt solution and another into No. 2 depression which already contains one drop of salt solution (12) Reject the serum not required, wash out the pipette in normal salt solution and dry on blotting paper (13) Mix serum and salt solution in No. 2 depression and take up a quantum of the mixture with the pipette (14) Let fall one drop of this mixture into No. 3 depression and return the remainder to No. 2 depression Wash out and dry pipette as before (15) Mix serum dilution and salt solution in No. 3 depression (16) Transfer one drop of this new mixture to No. 4 depression and return the residue to No. 3 depression Wash out and dry pipette as before (17) Take up in the pipette a quantum of well mixed test erythrocyte suspension (18) Let fall 2 drops into No. 4 depression and in succession one drop into Nos. 3, 2 and 1 depressions (19) Reject the remainder of the erythrocyte suspension in the pipette (20) Mix well the contents of each depression, beginning with No. 4 and working up to No. 1 (21) Take up from No. 1 a sample of the mixture of serum and test erythrocytes in a vaccine capillary tube (22) Give the column a tilt upwards in the tube, plunge the end of the tube into a cylinder of plasticine and withdraw it quickly, by which manœuvre the end is plugged (23) Place the capillary thus sealed in the water bath with its upper open end above the surface of the water and note the time (24) Do likewise as rapidly as possible, with the mixtures in Nos. 2, 3 and 4 depressions and place in the water bath alongside the first (25) Watch for the occurrence of agglutination in the capillary tubes and record the time of its occurrence (26) Remove the tubes after 15 minutes and record final results of agglutination and dilution (27) Place the capillary tubes upright in a layer of plasticine (28) Keep overnight in ice chest and read again, after inverting the tubes

The points which are noteworthy in the technique are —

(1) The use of capillary vaccine tubes, (2) the use of the drop as unit of volume, (3) the saving of time which is thereby effected in setting up dilutions, (4) the return of the residue in pipette to its mixture after the unit of volume has been subtracted from it, (5) the use of plasticine to close the capillary tubes, (6) the continuous observation of the reagent mixtures in a glass water bath, and (7) the employment of a time unit as measure of degree of reaction

## RESULTS

The five groups which emerged from the testing of each individual against each to begin with are numbered 1, 2, 3, 4 and 5, they are not intended, so far as these numbers go, to correspond with the Moss or Jansky groups In column I are exhibited the results of testing the sera of these 5 individuals against the erythrocyte suspensions of a series of 58 individuals, while in column II are shown the results of tests of the sera of the same 58 individuals against the erythrocytes of the selected

five. The grouping resulting from the double test is given in column III. Time to agglutination is given only for the highest dilution of serum in which it occurred. Thus 16.6 means that agglutination occurred in 6 minutes, while a simple 0 means that no agglutination was shown by even a twofold dilution in 15 minutes and of course none with any greater dilution within this time. As has already been pointed out, the research after the five original groups had been sorted out, resolved itself into a demonstration of degree of variability of agglutination only. This is demonstrated and suggests the conclusion that grouping might on this basis alone, give a more varied subdivision than even the five groups with which I started. The same type of conclusion is reached by others who have discovered subgroups to the main blood groups. A similar judgment has been given by Owen (1928) who deals with the possibility of the indefinite extension of the numbers of blood groups and with the time factor in agglutination.

## CONCLUSIONS

1. Five blood groups have emerged by testing the blood of a limited number of individual strictly against every other instead of by means of a standard serum. There is then a possibility that still more groups might be obtained if this method of testing were carried out.

2. The gradation of agglutination found within the groups, as judged by using a time unit as measure, points to the possibility of one group merging into another and to the possible existence of variant individuals of whom it cannot be said positively whether they belong to one or other of the selected groups or to a new group.

TABLE

*Showing blood grouping and degrees of agglutination with time as the unit of measure*

I						II					III
Erythrocytes of individuals						Sera of individuals					Group

TABLE—contd

I						II					III		
Erythrocytes of individuals						Sera of individuals					Group		
	1	2	3	4	5		1	2	3	4	5		
Sera	7	16 7	16 10	16 6	0	8 15	Erythrocytes	0	0	0	0	0	4
	8	16 10	8 15	16 12	0	4 12		0	0	0	0	0	4
	9	16 4	16 6	16 2	0	8 15		0	0	0	0	0	4
	10	0	0	0	0	0		2 15	0	16 8	16 15	0	2
	11	16 15	2 10	16 9	0	0		0	0	16 10	4 10	0	5
	12	16 2	2 12	16 6	0	0		0	0	16 5	8 12	0	5
	13	8 6	2 12	16 6	0	0		0	0	16 3	8 9	0	5
	14	8 13	4 6	2 2	0	4 12		0	0	0	0	0	4
	15	4 15	0	4 15	0	0		2 15	0	16 3	16 8	0	2
	16	4 7	0	4 10	0	0		2 15	0	16 10	16 15	0	2
	17	16 12	5 7	16 11	0	16 12		0	0	0	0	0	4
	18	8 14	0	1 2	0	0		2 6	0	16 2	16 3	0	5
	19	16 10	0	16 15	0	0		0	0	16 3	16 15	0	5
	20	0	16 3	0	0	16 6		0	0	0	16 5	8 4	3
	21	8 15	0	8 10	0	0		2 15	0	16 4	16 12	0	5
	22	16 6	0	16 12	0	0		2 15	0	16 4	16 13	0	5
	23	4 8	0	8 10	0	0		0	0	16 4	16 15	0	5
	24	8 15	0	8 10	0	0		0	0	16 5	8 10	0	5
	25	16 10	2 15	16 12	0	0		0	0	16 4	8 10	0	5
	26	0	16 8	0	0	16 6		0	0	0	16 10	16 12	3
	27	16 11	0	8 10	0	0		0	0	16 8	4 6	0	5
	28	0	16 7	0	0	16 12		0	0	0	16 7	8 15	3
	29	4 5	0	4 12	0	0		0	0	16 10	8 15	0	2
	30	4 12	0	4 15	0	0		0	0	16 8	8 15	0	2
	31	8 8	0	16 15	0	0		2 10	0	16 5	8 11	0	5
	32	16 4	16 8	16 9	0	16 10		0	0	0	0	0	4
	33	0	0	0	0	0		0	0	16 8	16 10	4 15	2
	34	16 4	16 10	16 3	0	16 10		0	0	0	0	0	4

TABLE —*concl'd*

	I					II					III	
	Erythrocytes of individual					Sera of individuals					Group	
	1	2	3	4	5	1	2	3	4	5		
Seri	15	16.5	16.10	16.15	0	16.11	0	0	0	0	0	4
	16	0	1.9	0	0	1.10	0	0	0	16.1	16.15	3
	17	0	0	0	0	0	0	0	0	16.7	8.3	1
	18	1.10	0	2.5	0	0	2.15	0	16.5	1.15	0	2
	19	16.2	16.15	16.2	0	1.8	0	0	0	0	0	4
	20	16.3	16.10	16.7	0	8.15	0	0	0	0	0	4
	21	16.15	1.10	16.10	0	8.15	0	0	0	0	0	1
	22	0	16.10	0	0	16.9	0	0	0	16.13	8.12	3
	23	16.7	0	16.6	0	0	1.12	0	16.5	8.7	0	5
	24	16.3	16.1	16.5	0	16.10	0	0	0	0	0	1
	25	16.10	0	16.7	0	0	0	0	16.1	8.4	0	0
	26	16.7	0	16.7	0	0	1.15	0	16.6	16.15	0	5
	27	1.15	0	8.15	0	0	2.7	0	16.13	16.12	0	5
	28	8.10	0	8.12	0	0	2.8	0	16.12	8.13	0	5
	29	16.5	1.15	16.8	0	0	2.15	0	16.8	1.12	0	5
	30	1.10	8.13	1.13	0	8.10	0	0	0	0	0	4
	31	0	8.15	0	0	1.9	0	0	0	16.6	16.12	3
	32	16.10	0	16.12	0	0	4.8	0	16.8	16.15	0	5
	33	16.10	16.8	16.7	0	8.5	0	0	0	0	0	4
	34	16.1	16.7	16.8	0	16.8	0	0	0	0	0	4
	35	0	0	0	0	0	2.6	0	16.10	8.10	0	2
	36	16.11	1.10	16.5	0	0	2.8	0	16.12	8.5	0	5
	37	16.6	2.15	16.12	0	0	0	0	16.10	8.15	0	5
	38	0	8.8	0	0	8.10	0	0	0	16.5	16.10	3

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Some Observations on Blood Grouping *Edin Med Jour*, Vol XXXV, p 222



# SOME OBSERVATIONS ON THE ULTRA-VIOLET INTENSITY IN CALCUTTA

BY

A. T. MUKHERJEE, M.Sc., T. C. BOYD, I.I.C., B. K. BOSE, M.Sc.  
(*Department of Chemistry, Medical College, Calcutta*)

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For all practical purposes the ultra violet region of the spectrum may be said to commence at about 3900 Ang. units. To determine the actual extent in the solar spectrum in Calcutta we carried out observations using a Hilger quartz spectrograph and Ilford panchromatic plates. One of these exposures showing a copper spectrum in juxtaposition is shown in Plate LXXVII, fig. 1.

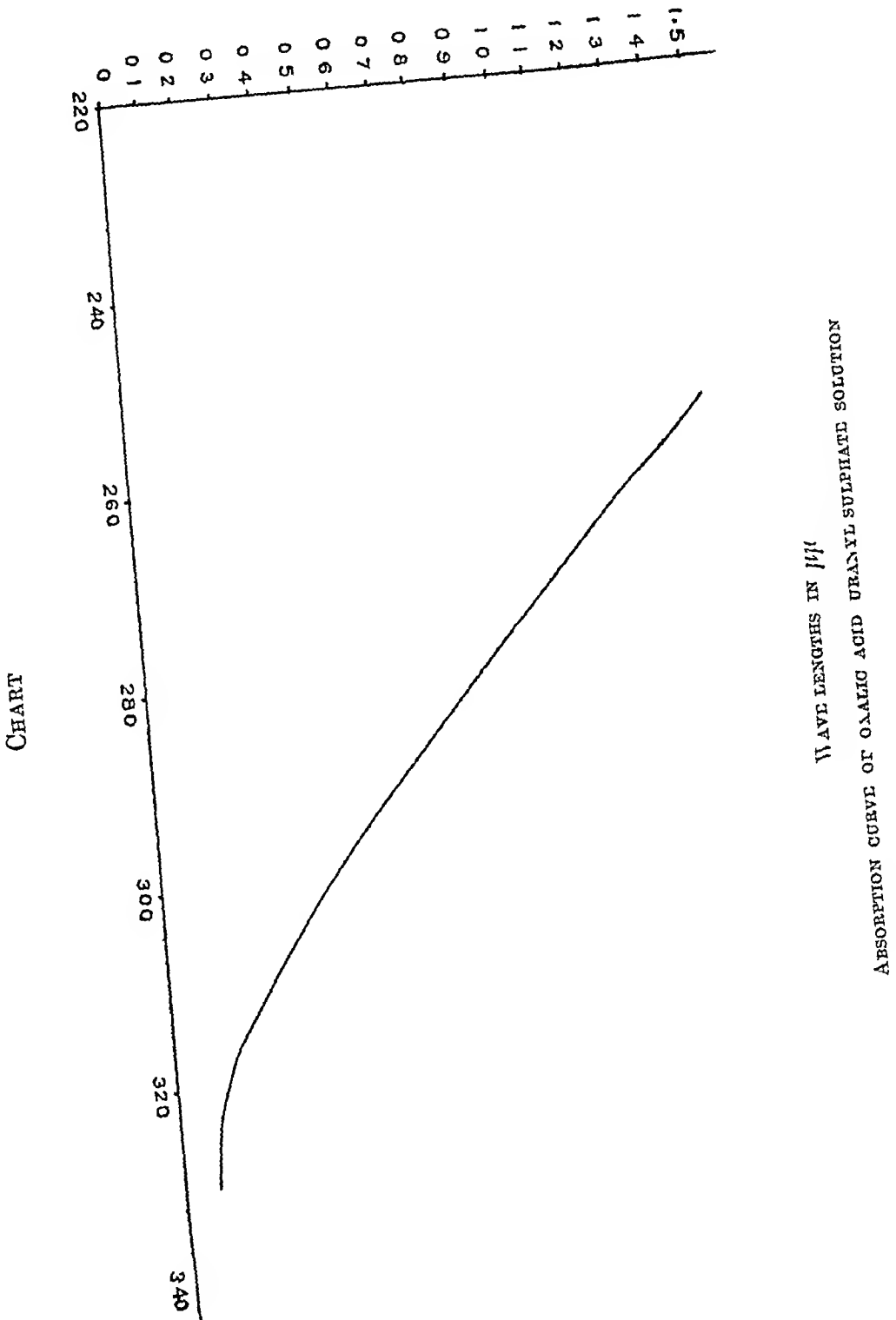
It will be seen that to obtain the actual limitation is extremely difficult but it may be considered to extend to about 2950 Å.

Our next step was to select a method suitable for the measurement of the ultra-violet intensity and we decided to adopt the method described by Leonard Hill in the *Lancet* (1924, 1, 715). This consists of determining the degree of bleaching of an aqueous acetone solution of methylene blue. Unfortunately, however, on correlating some of our results we found many discrepancies. These on further investigation appeared to be due to variations caused by different brands of acetone. We were thus forced to abandon this method and the results obtained. We next turned our attention to the Anderson Robinson oxalic acid uranyl sulphate ultra-violet radiometer (*Jour Amer Chem Soc*, 1925, 47, 718). This in principle depends on the photo chemical decomposition of oxalic acid sensitized by uranyl sulphate. The actual solution used consisted of exactly 6.3 grammes of pure oxalic acid and 4.2 grammes of uranyl sulphate, dissolved in one litre of distilled water. Ten c.c. of this solution were exposed in quartz and glass test tubes of the following dimensions —

Quartz tube Internal diameter = 1.87 cm, height of solution = 4.19 cm,  
approximate surface = 27.3 sq. cm. or 4.23 sq. inches

Glass tube Internal diameter = 1.86 cm, height of solution = 4.2 cm,  
approximate surface = 27.2 sq. cm. or 4.23 sq. inches

( 1313 )







# PLATE LXXVII



FIG 1.

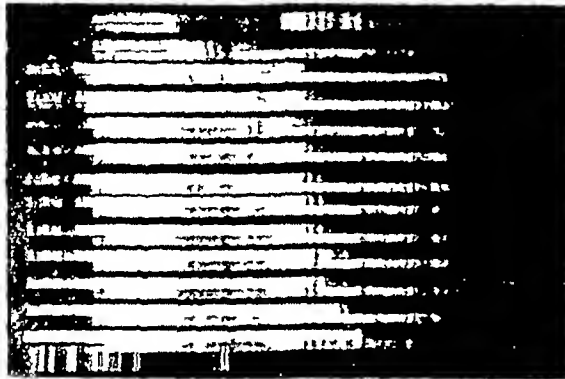


FIG. 2.

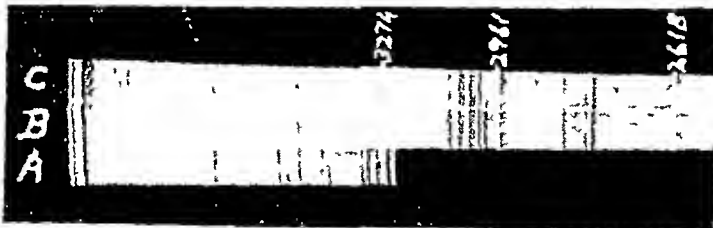


FIG 3

The solutions were subsequently titrated with 0.1 N potassium permanganate solution and the result expressed in milligrams of oxalic acid decomposed. The actual absorption curve of the methyl oxalic acid solution obtained by using a Hilger rotating disc sector photometer is given (Plate LXXVII fig. 2).

The curve obtained closely resembles that given by Gillam and Morton (*J. S. C. I.* Nov. 1927, 115 T) and is noted by them there is by no means a negligible absorption in the region 300–350  $\mu$ . Further the above workers found that from 25 to 30 per cent of the decomposition occurs with rays on the long wave side of 325  $\mu$ , about 40 per cent with rays on the long wave side of 280  $\mu$ , while the region 270–326  $\mu$  accounts for about another 20 per cent. These wave lengths and their effects are very important because according to Cumberbatch's paper (*Brit. Med. Jour.* July 1911, 28, 15) wave lengths 310 to 390  $\mu$  have probably no chemical action on the skin, while wave lengths 290 to 330  $\mu$  have pigment producing effects. Hesse also notes that 305  $\mu$  is very important in the treatment of wickets.

To show the actual rays absorbed by the test tubes a spectrograph is given (Plate LXXVII fig. 3) in which A gives the absorption of the glass test tube, B shows the absorption of the quartz test tube while C is the copper spectrum.

In the above spectrograph it may be seen that the quartz test tube has practically no absorption while absorption begins in the glass tube about 330  $\mu$  and as the solution was exposed in sunlight in glass and quartz tubes the difference between the readings of the two is a measure of the intensity of the region from about 330  $\mu$  to 295  $\mu$ .

Before actually working with these tubes in sunlight we made some observations using a Hanovia incandescent mercury vapour lamp working on a 220 D.C. current. Some of these experiments may be cited as they supply to some extent a link between the decomposition of the methyl sulphate oxalic acid solution and the range of exposure liable to produce an erythema in clinical practice.

(1) Distance of tubes from lamp = 12 inches, current 3 amps, 10 c.c. of methyl oxalic acid solution, surface exposed 13.7 sq. cm. or 2.12 sq. inches, time = 30 minutes

Quartz tubes	=	11.7	milligrams of oxalic acid decomposed
Glass tube	=	1.1	do do do do

Therefore the decomposition due to rays shorter than 330  $\mu$  is equal to 7.3 milligrams of oxalic acid.

(2) Usually, however, clinical exposures to the mercury vapour lamp are given at a distance of three feet. Using this distance and working with the lamp at 3 amps, we obtained the following results with the above mentioned tubes —

Exposure	Glass tube	Quartz tube
5 minutes	Nil	Nil
15 minutes	Nil	0.3 mg
30 minutes	0.6 mg	1.6 mg

The findings agree approximately with those found in experiment (1), when calculated on the basis of the law of inverse squares. The *ml* findings may possibly be accounted for by supposing a period of induction for the reaction. Cumberbatch (*ibid*) describes the following clinical method of administering ultra-violet treatment. The patient, stripped except the genitalia, is exposed horizontally to the lamp suspended three feet above the middle of the body and a little to one side. The first exposure consists of two minutes back and front and the exposures are repeated every alternate day until he receives ten minutes back and front. It is of interest to calculate approximately the surface area exposed and translate the result into milligrams of oxalic acid that would be decomposed on such a surface. To do this we placed an average sized Indian lad 5 feet 5 inches in height on a bench and suspended a lamp three feet above the lumbar region and a little to one side, and proceeded to evaluate roughly the area that was exposed to the rays. The back area thus calculated amounted to about 640 sq inches, the front area would be approximately the same, say a total area of about 1,280 sq inches or 9 square feet, an area roughly 604 times the area of the surface exposed in the glass and quartz tubes. An exposure on this surface for fifteen minutes ( $7\frac{1}{2}$  minutes back and front) would correspond to something in the vicinity of 302 milligrams of oxalic acid, decomposed due to rays shorter than 330  $\mu$  or to about 33 milligrams of oxalic acid per square foot of surface exposed for fifteen minutes.

For our sun observations the tubes glass and quartz, were exposed at right angles to the sun's rays for 30 minutes (from 1 P M to 1-30 P M) on the roof of the School of Tropical Medicine and Hygiene, Calcutta, the highest building in the immediate neighbourhood, commanding a good view of almost the whole of the horizon. This condition is necessary as according to Hill (*Trans Royal Soc*, Nov 1927), the ultra-violet intensity of the blue sky only bears a fair proportion to the total ultra-violet intensity. The results of these readings showing the day of the month and the condition of the sky at the time of exposure are shown in the attached graphs (Graphs 1 to 12).

It may be noted that, though the intensity of the extreme ultra-violet is somewhat dependent on the total ultra-violet intensity of the sunlight as measured by the decomposition of oxalic acid in the quartz tube it is not strictly proportional to the same, other factors like dust and the amount of ozone in the upper layers of the atmosphere (Harrison, *Nature*, July 13th, 1929) have to be considered. It will be observed that the intensity of the extreme ultra-violet region as well as the total ultra-violet intensity though not very high, is almost constant throughout the winter months from November to January after which period it gradually rises until the beginning of the Monsoon when it becomes exceedingly fluctuating (Graph 15). Another point that is of interest is the increase of the ultra-violet intensity of the sunlight after a heavy shower of rain, a probable explanation for the burning of the skin often experienced on rainy days with periods of bright sunshine.

In addition to carrying out these monthly observations we have also taken two half-hourly readings throughout the day selecting as far as possible days that exhibited an absolutely clear sky. These results are shown in graphs (Graphs 13 and 14) the curves obtained are parabolic in form and show the maximum intensity between 12 noon and 12.30.

It is of interest to compare as far as possible the quantity of the ultra-violet radiation as determined at Bourville in bright sunshine on July 2nd, 1926, as observed by Moss and Knapp (*Brit. Jour. of Actinotherapy* May 1927-37), using their modified 'Twenty strength' solution and apparatus to that in the sunlight in Calcutta. Translating Moss and Knapp's readings we got 31 per cent decomposition of the 'Twenty strength' solution in fifteen minutes on a surface of 15 cms diameter equivalent to a decomposition of 22.19 mg. of oxalic acid in 30 minutes, in a quartz tube of the size used by us. The maximum decomposition noted by us in Calcutta on the 1st May amounted to 40.5 mg. of oxalic acid decomposed in 30 minutes, almost double that obtained in Bourville.

We are now in a position to compare on an oxalic acid decomposition basis, the effects of exposure to the mercury vapour lamp with that obtained by exposure to the sunlight at Calcutta. Taking 5 mg. of oxalic acid as the average difference between the decomposition in the quartz and glass tubes on a clear day in summer on a surface of 1.23 sq. inches in thirty minutes and 1 milligram of oxalic acid as the difference in the case of the mercury vapour lamp on a surface of 2.12 sq. inches for the same period, an average exposure to the sun on any clear day in summer at noon for thirty minutes is equivalent to two and a half times the exposure to our lamp at a distance of three feet for 30 minutes so far as the region shorter than about 3,300 ÅU is concerned.

#### SUMMARY AND CONCLUSIONS

- (1) The lower limit of the solar spectrum at Calcutta has been approximately noted.
- (2) Hill's acetone methylene blue method has been found to yield discrepancies with different brands of acetone.
- (3) The intensity of the ultra-violet rays in the sunlight of Calcutta has been studied using the Robinson Anderson uranium oxalic acid radiometer, over a period of twelve months and the variations recorded.
- (4) The equivalent oxalic acid decomposition figure has been compared to the probable average erythema dose.
- (5) It has been shown that the period of maximum intensity of the ultra-violet light of the sun at Calcutta is between noon and twelve-thirty.
- (6) The intensity of the physiologically active rays in sunlight at noon in Calcutta is found to be at least two and a half times that of a mercury vapour lamp.

# EXPLANATION OF GRAPHS

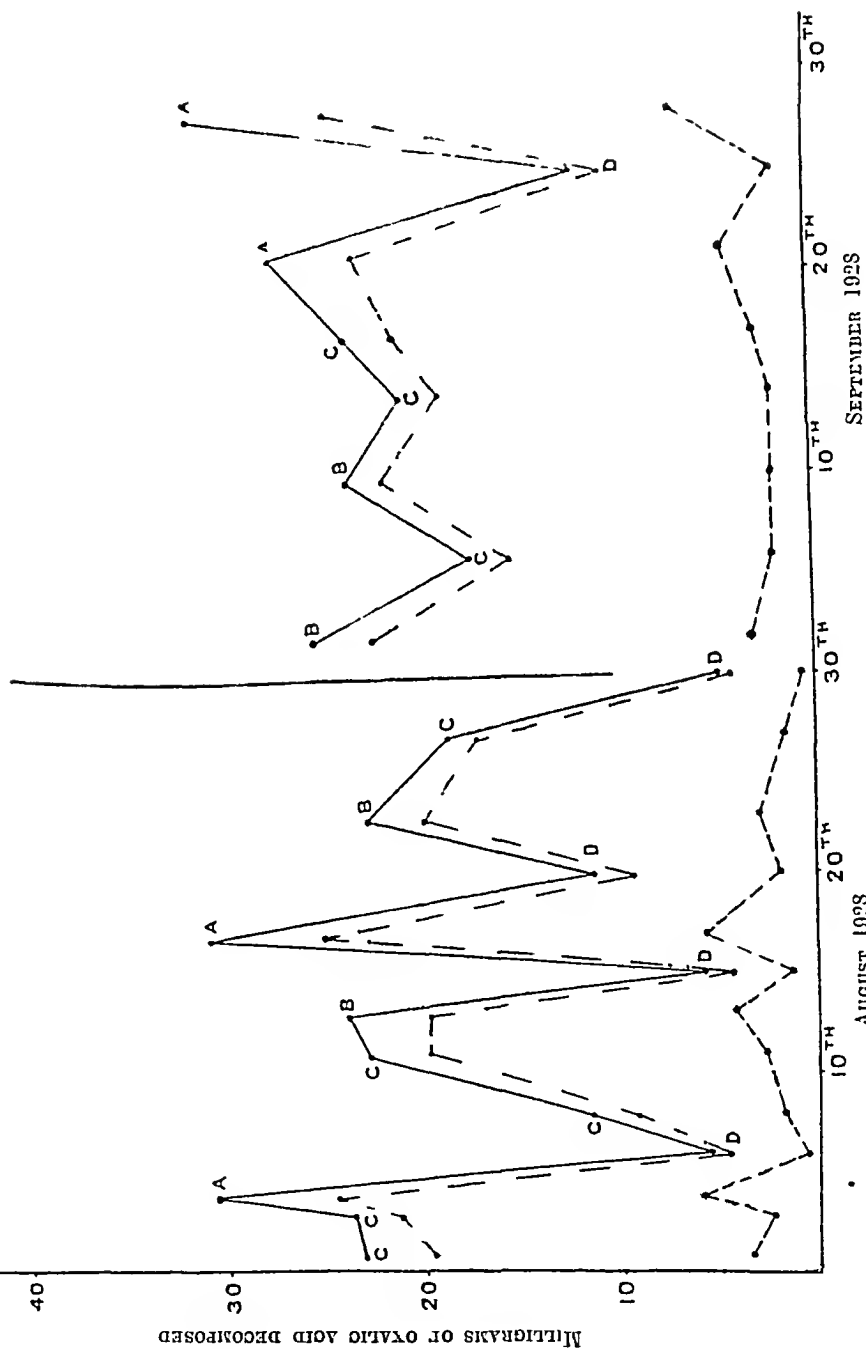
The whole line ( ————— ) shows the decomposition in the quartz test tube (i.e., the total ultra-violet intensity)

The chained line ( — — — — — ) shows the decomposition in the glass test tube (i.e., the intensity of rays on the long wave side of about 3,300 AU)

The broken line ( — — — — — ) shows the difference between the above two giving the intensity of rays between about 3,300 AU and 2,950 AU (i.e., the intensity of the extreme ultra-violet rays present in the sunlight in Calcutta)

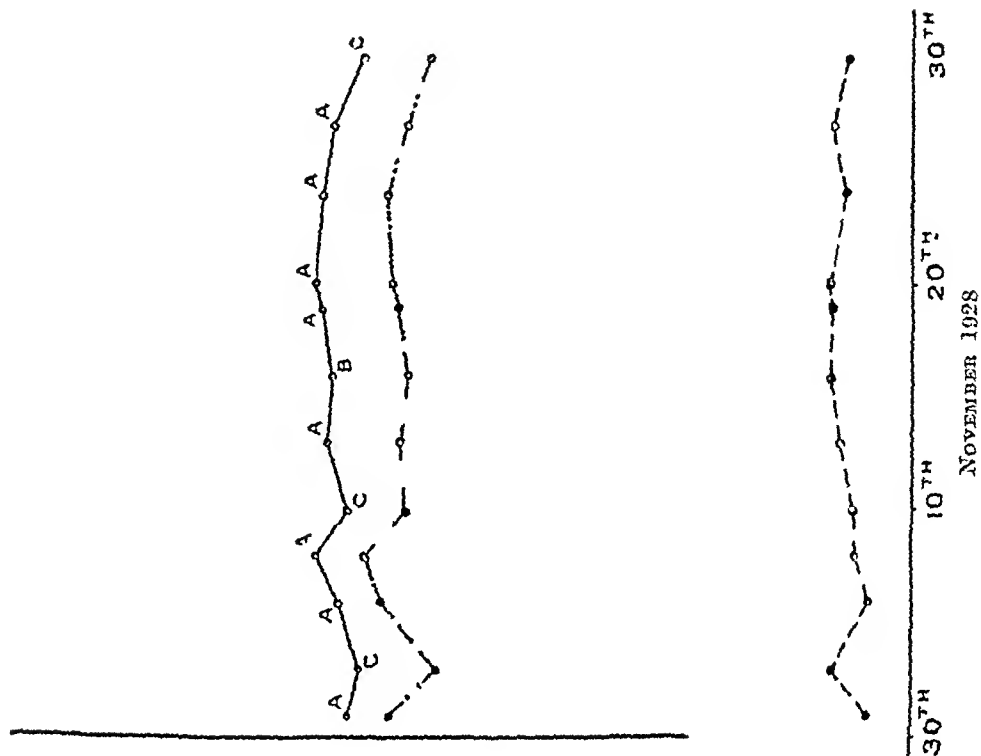
- A Bright sun—clear sky
- B Bright sun—cloudy sky
- C Hazy sun
- D Cloudy sun

GRAPH 2

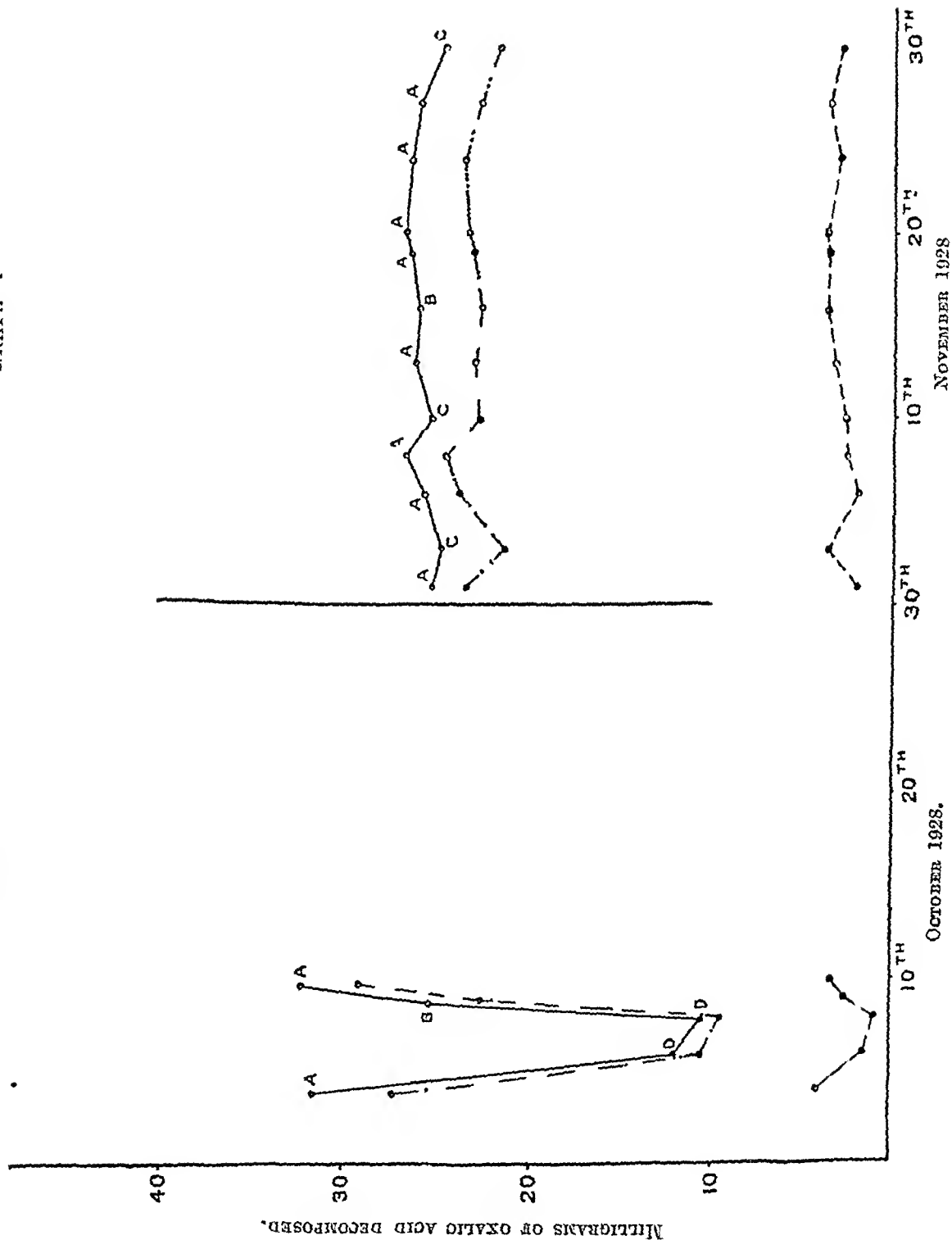


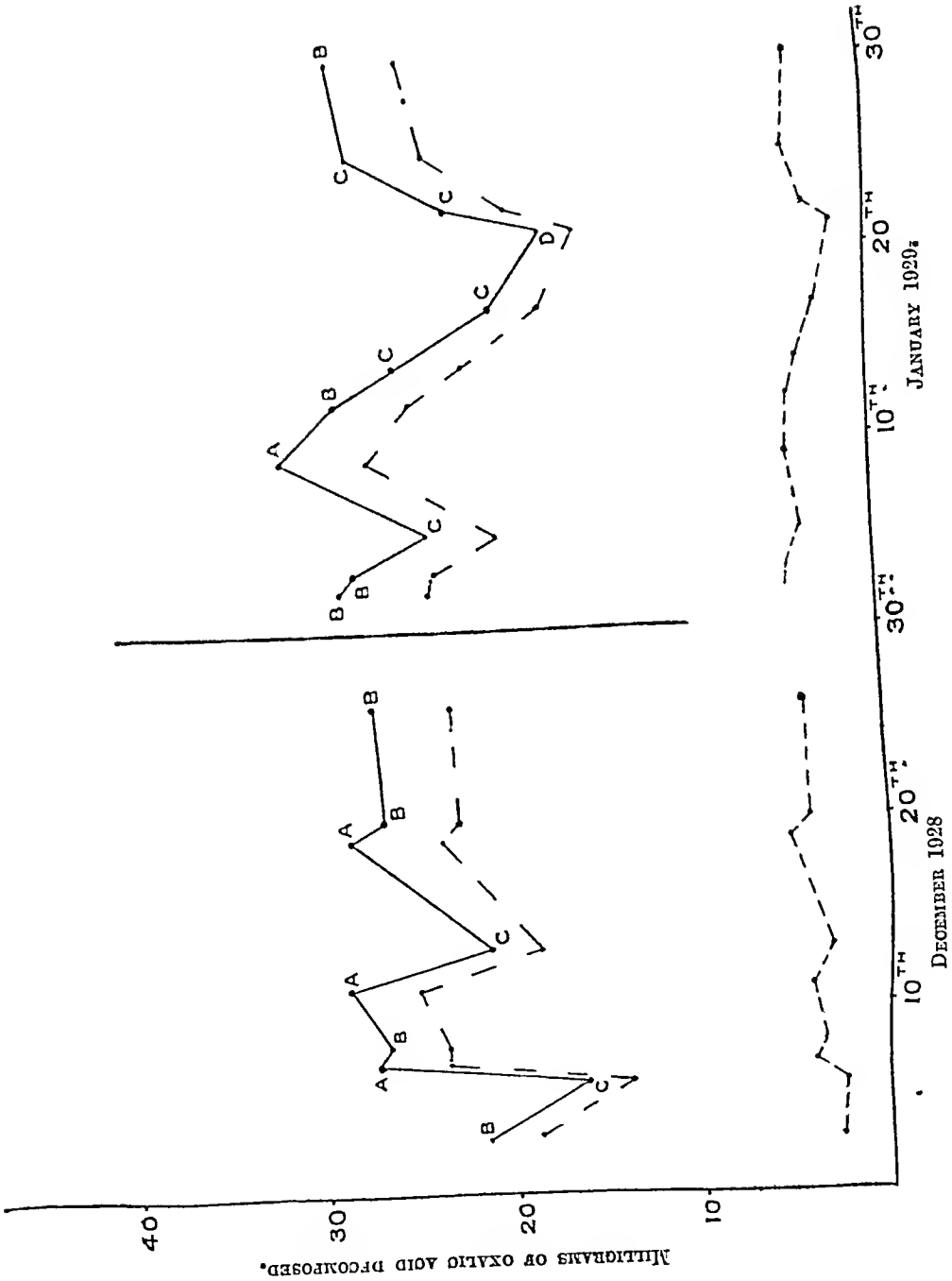
GRAPH 1

GRAPH 4



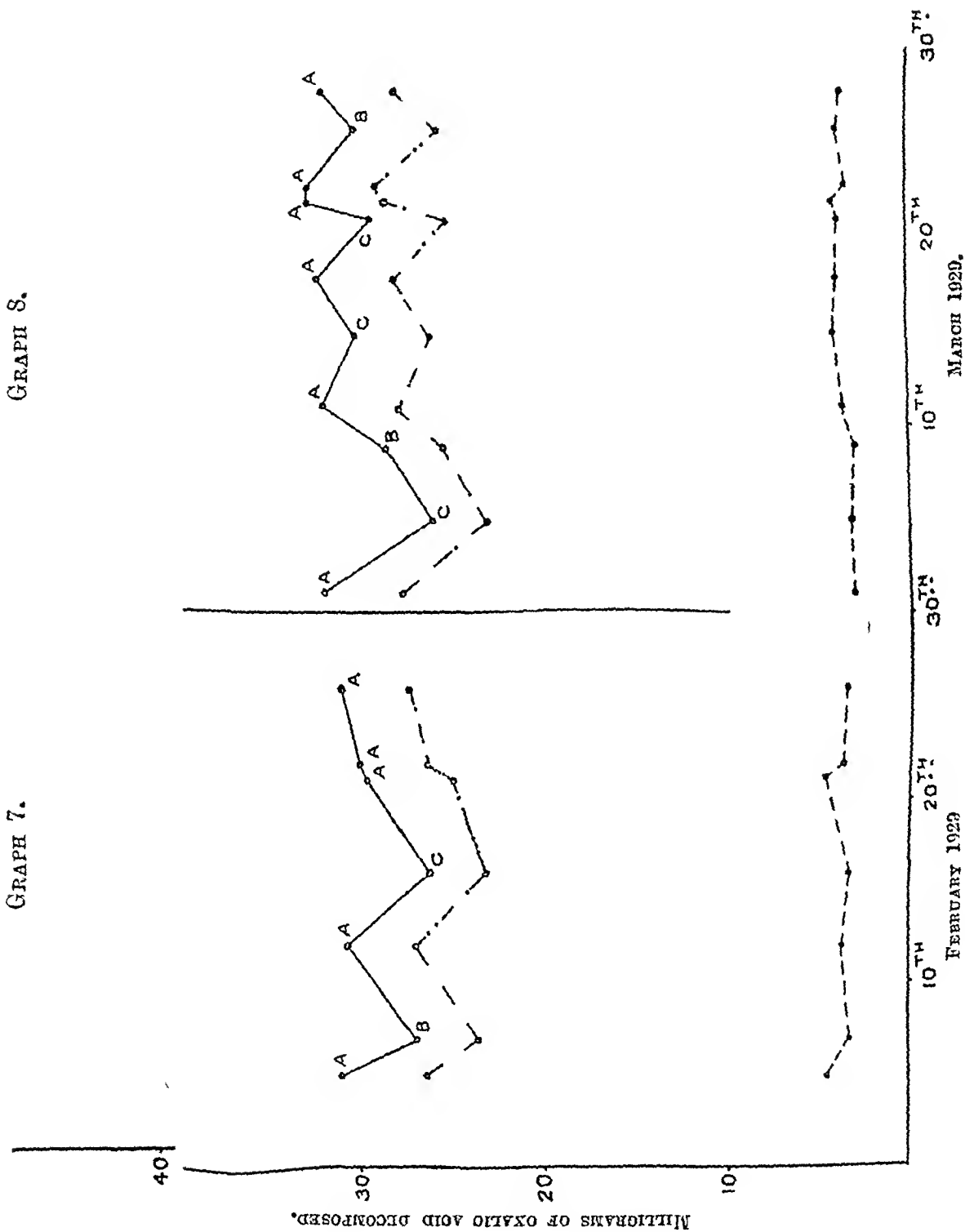
GRAPH 3.



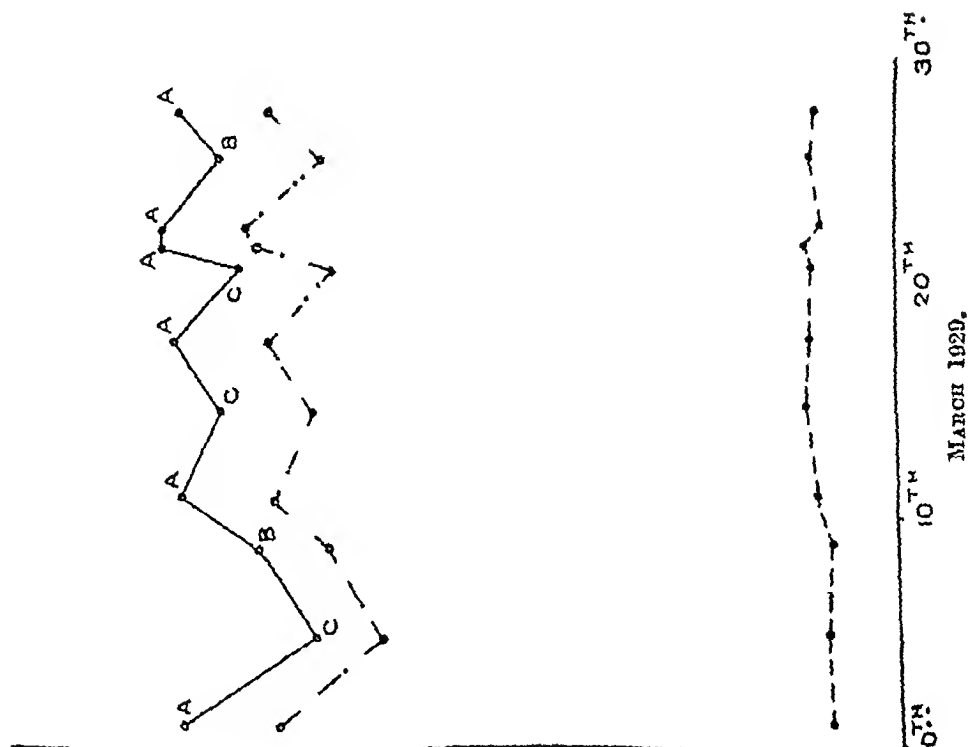




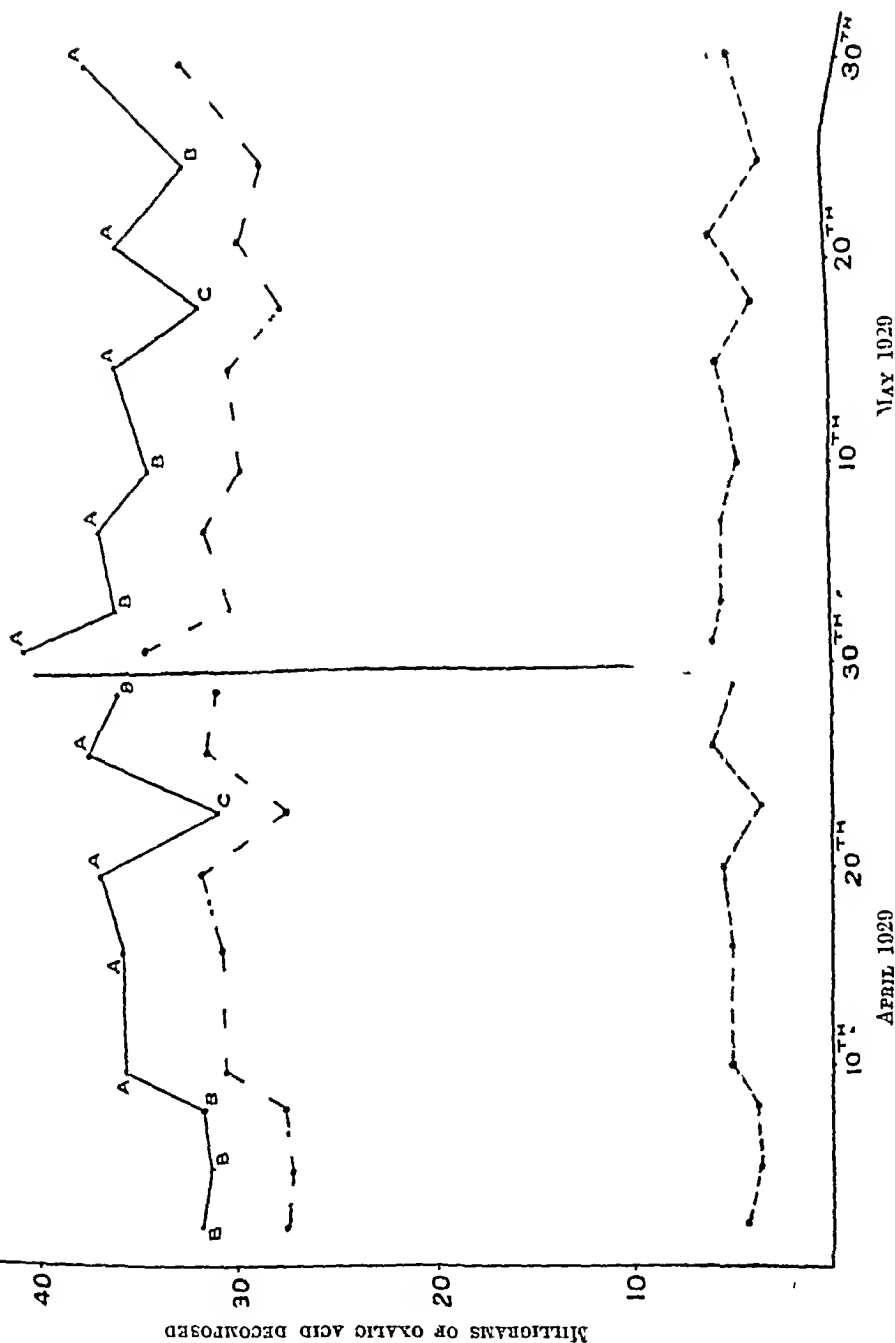
GRAPH 7.



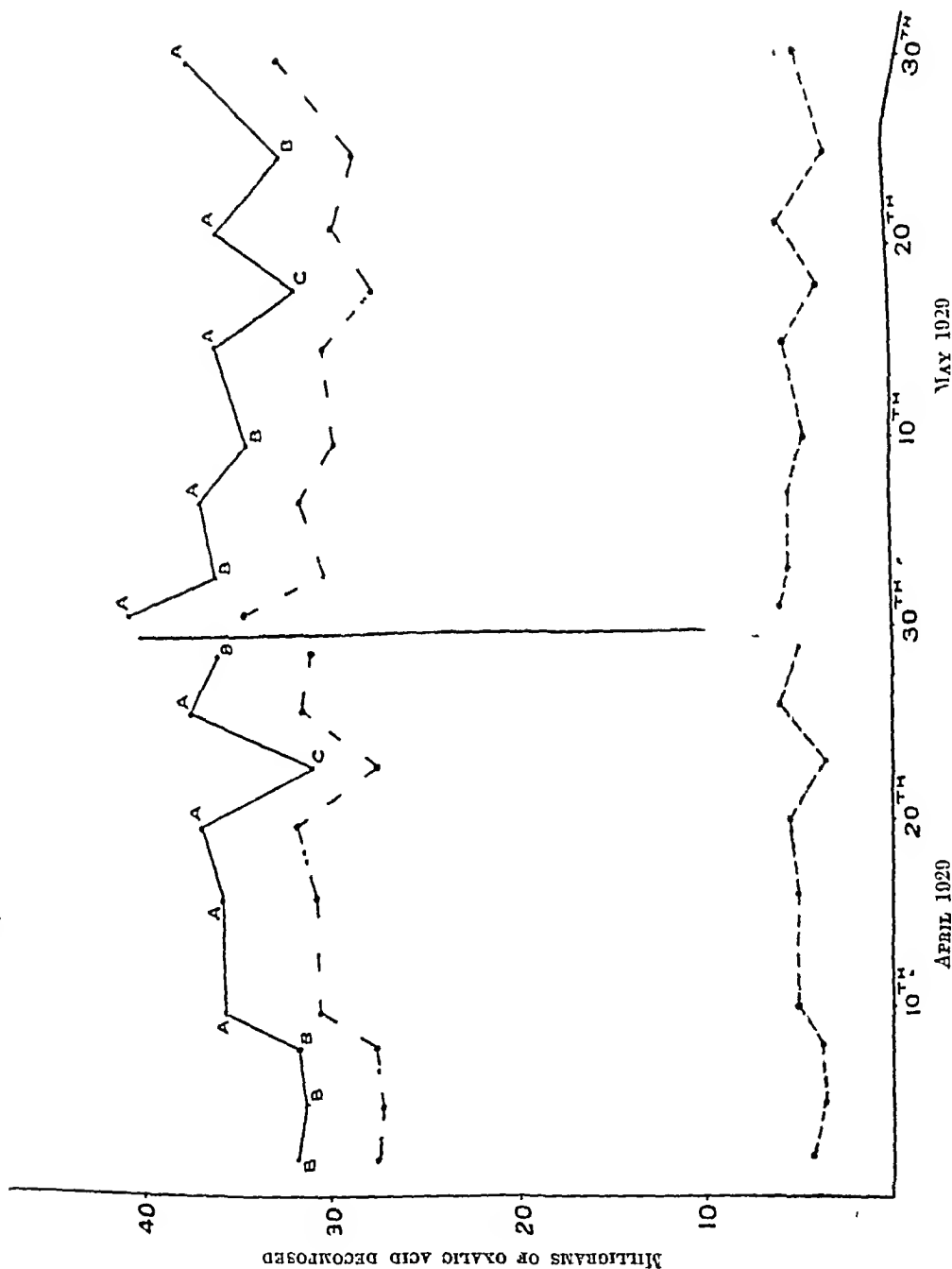
GRAPH 8.



GRAPH 10

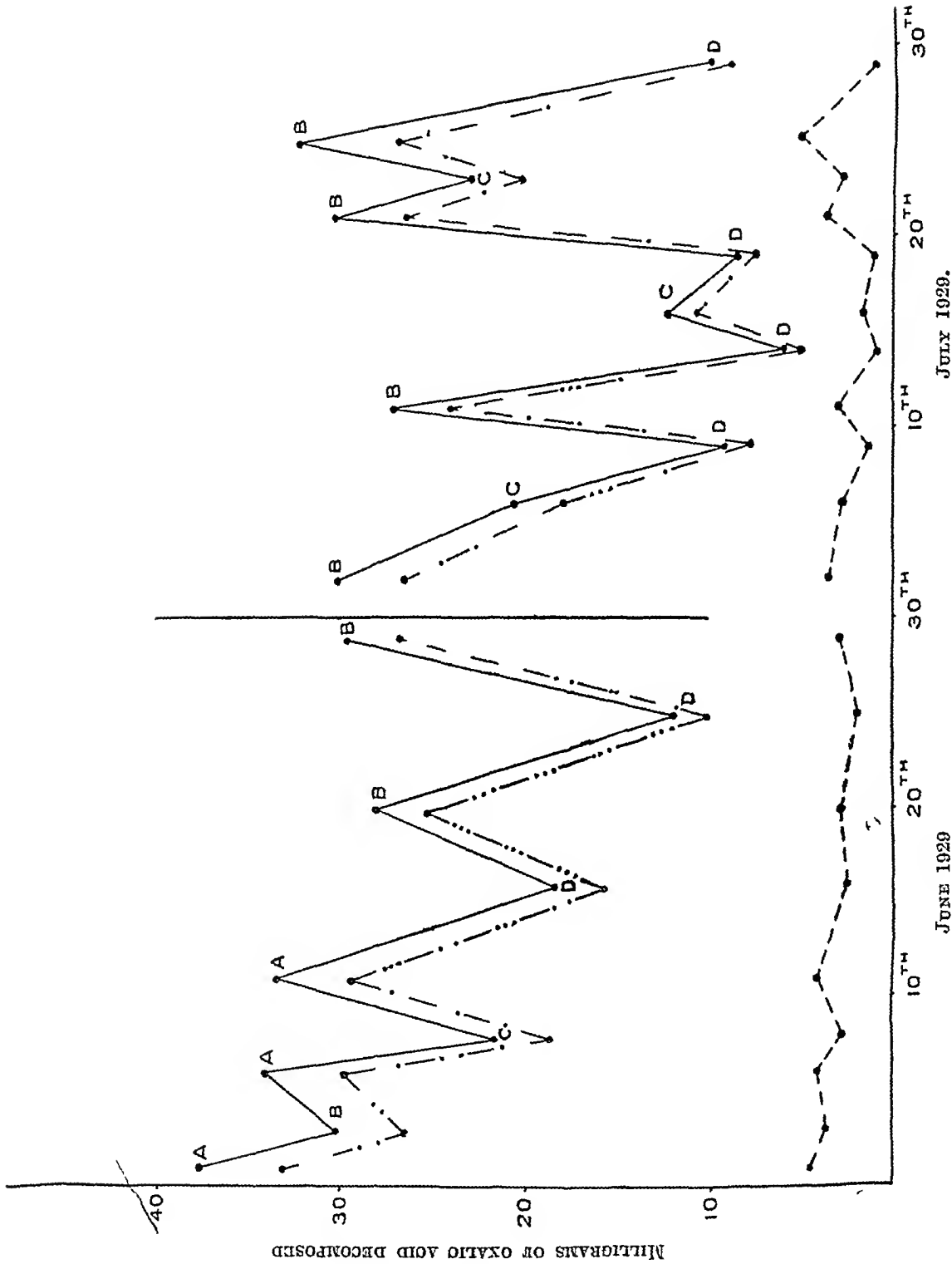


GRAPH 9.

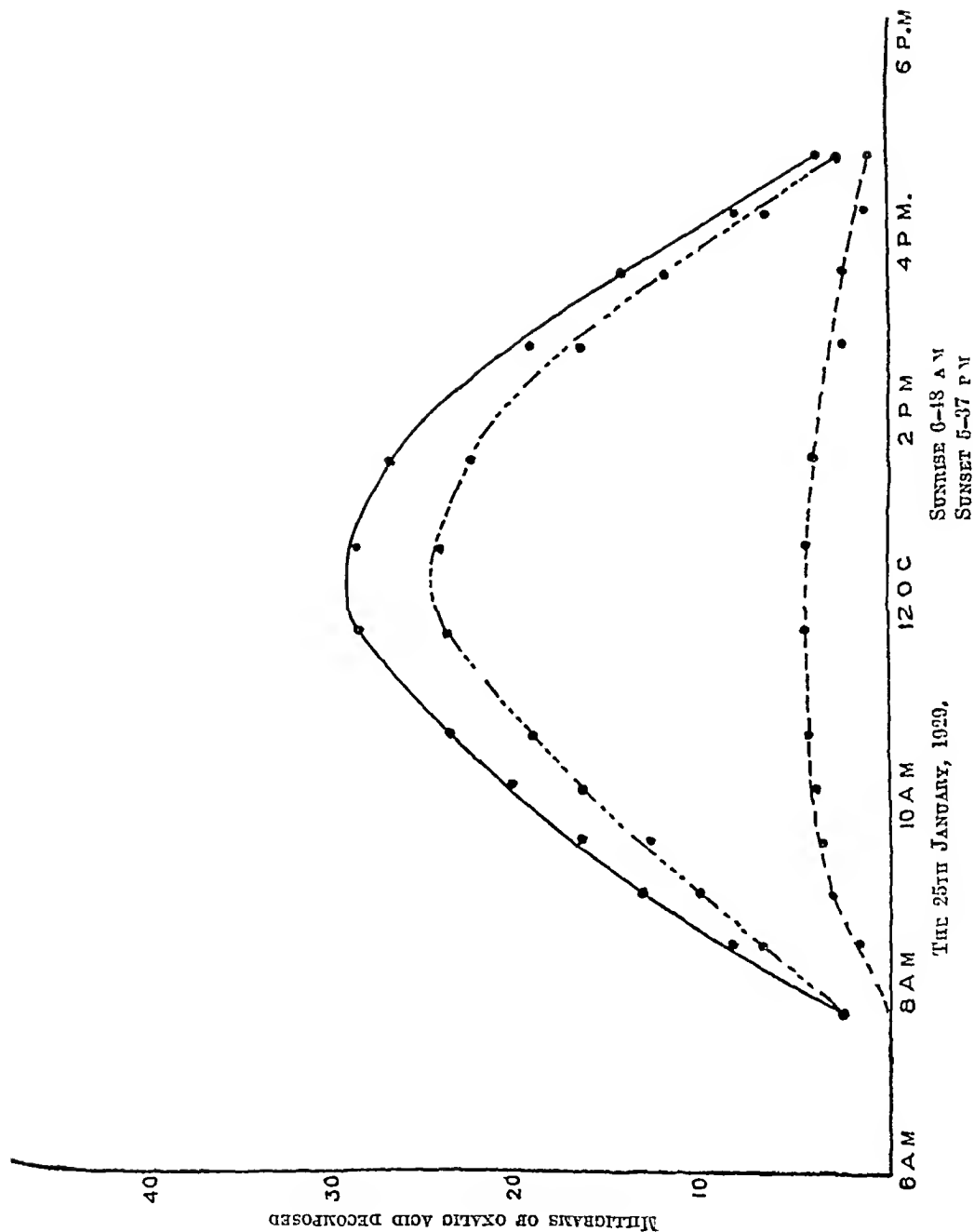


GRAPH 12.

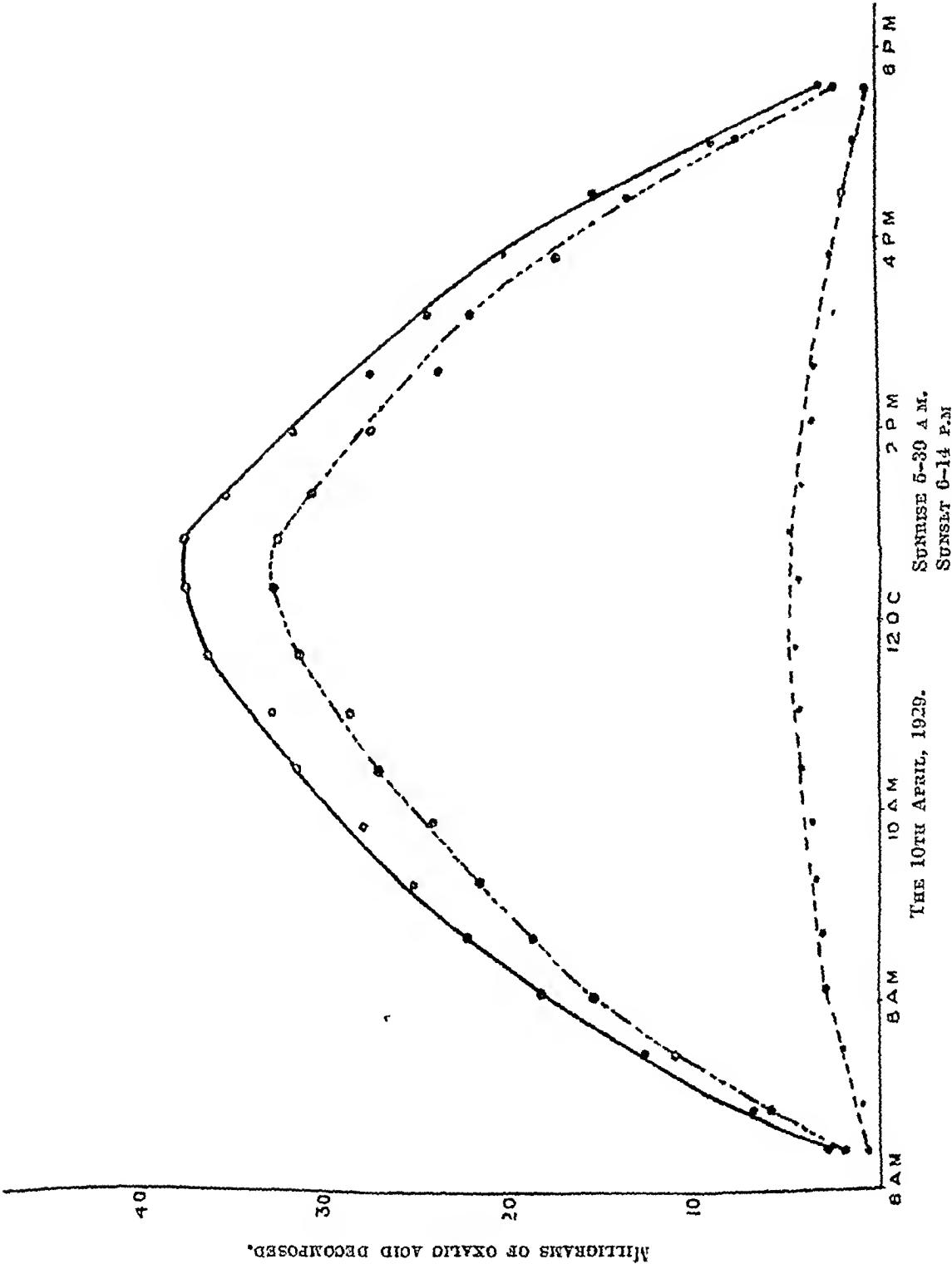
GRAPH 11



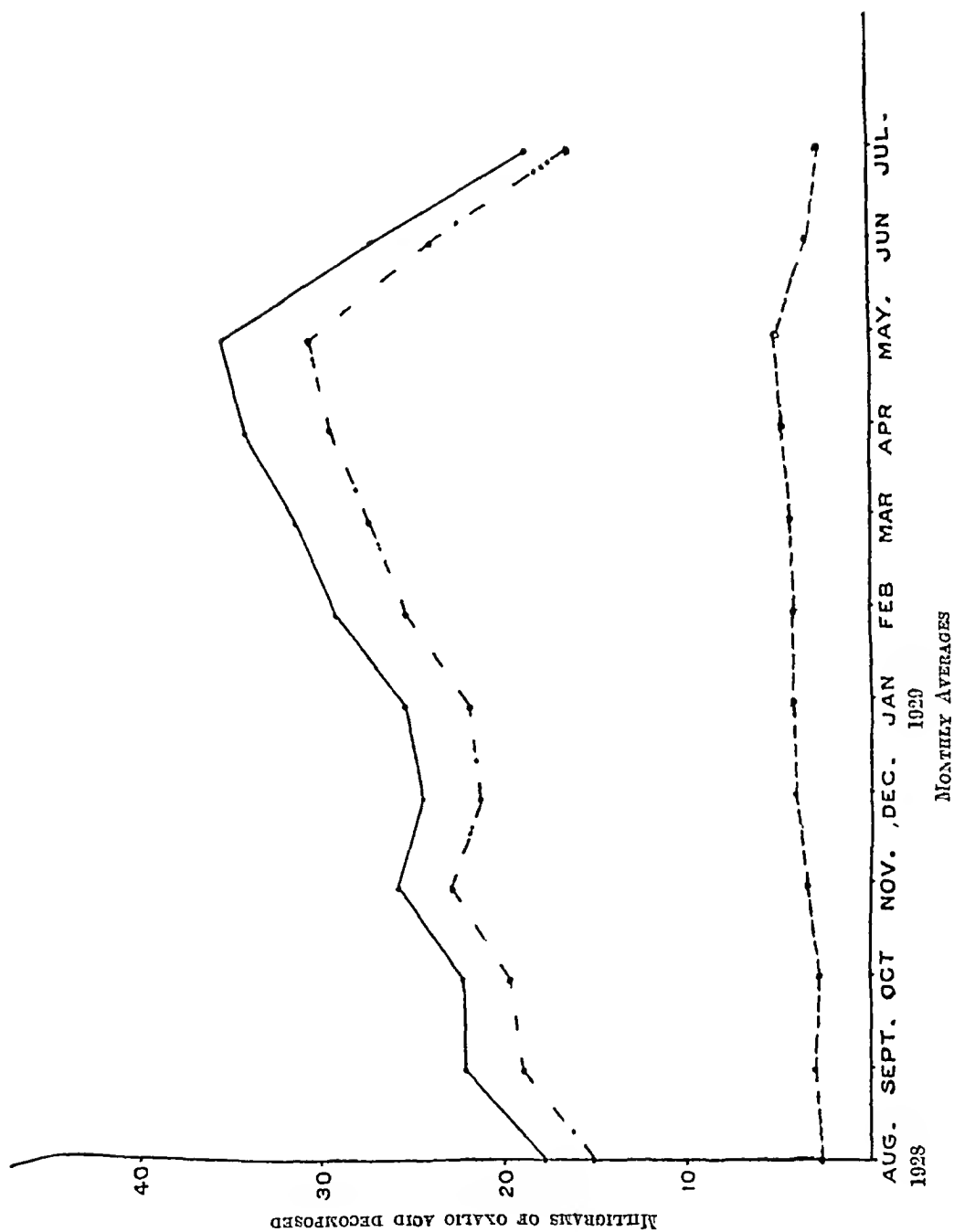
GRAPH 13



GRAPH 14



GRAPH 15



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